Simple Adult Rabbit Model for *Campylobacter jejuni* Enteritis

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We tested the usefulness of the Removable Intestinal Tie Adult Rabbit Diarrhea model to establish *Campylobacter jejuni* infection in rabbits. The procedure involved ligation of the cecum, placement of a slip knot at the terminal ileum, and injection of the test inoculum into the mid-small bowel. The ends of the slip knot were externalized, and the tie was released 4 h later. Fifty-five rabbits received *C. jejuni*, and 16 received uninoculated medium as controls. Daily rectal swabs were positive for 2 weeks in infected rabbits. The diarrheal attack rate was 64% in infected rabbits and 0% in controls. Diarrhea was characterized by loose, mucus-containing stools after an incubation period ranging from 24 h to 6 days. When blood was obtained daily for culture from 30 rabbits for 4 days post-challenge, bacteremia was present in 96.3% 24 h after challenge but diminished to 5 of 19 (26.3%) at 96 h. Death occurred in 53% of rabbits and was always preceded by diarrhea. No control animal died. Only 5 of 35 animals experiencing diarrhea recovered. An indirect whole-cell enzyme-linked immunosorbent assay was used to determine serum immunoglobulin G responses. Mean titers rose from 1:198 preoperatively to 1:9,087 on day 28. Necropsy on eight infected and two control animals showed inflammatory lesions with ulceration in 62.5% and goblet cell hyperplasia in 75% of infected rabbits. We conclude that the Removable Intestinal Tie Adult Rabbit Diarrhea procedure is a simple, effective method to establish *C. jejuni* infection which mimics human disease.

*Campylobacter fetus* subsp. *jejuni* (C. *jejuni*) is a major worldwide cause of bacterial diarrhea and in some studies is the most frequent bacterial pathogen isolated from stool (4, 6, 19). Human infections are usually self-limited, lasting 3 to 5 days beyond an incubation period of 2 to 7 days (4). Mucous stools, sometimes containing blood, are characteristic of *Campylobacter* diarrhea (1, 6). Rectal biopsies obtained from patients with *Campylobacter* infection often show colitis with edema of the lamina propria, focal mucosal infiltrates, and crypt abscesses (12, 17).

Little is known about the pathophysiology of *Campylobacter* infections. This is due in part to the failure to demonstrate significant effects with this organism, comparable to those seen with other enteric infections. *Campylobacter* induces no reactions in the classical model systems such as the Sereny test (11), suckling mouse assay (11, 15), and ligated rabbit ileal loops (11). These failures, and others using in vitro methods, have created a need for new in vivo models. Experimental *Campylobacter* infections have been attempted in mice (2), gnotobiota beagle puppies (16), chicks (18), ferrets (J. Ackerman, J. Fox, and J. Murphy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B143, p. 41), newborn calves and lambs (9), and monkeys (10). These models are unsuitable for extensive study because of expense and difficulty in animal care, or because the models fail to produce acute disease symptoms.

Spira et al. developed an adult rabbit model which resulted in diarrhea and death when *Vibrio cholerae* or enterotoxigenic *Escherichia coli* organisms were inoculated into the ileum proximal to a removable intestinal tie (20). A key factor in the Removable Intestinal Tie Adult Rabbit Diarrhea (RITARD) model is that colonization of the mucosa is enhanced through the temporary inhibition of normal peristaltic clearance mechanisms. Gut patency is restored after removal of the temporary ligation, thereby permitting time for the incubation period and the evolution of the infection.

We tested the usefulness of the RITARD method to establish infections with *C. jejuni* in rabbits. The disease produced was similar to human infections and may, therefore, be useful for the study of virulence mechanisms and host responses.

**MATERIALS AND METHODS**

**Bacterial strains.** Two strains of *C. jejuni* were used. Strain 39-3 RLS is an isolate from calf stool recovered from rabbit liver and spleen after preliminary studies.
with the RITARD method. Strain HC was isolated from the blood of an otherwise healthy patient with enteritis. Both strains were passaged twice on artificial media and then were maintained as frozen stock in liquid nitrogen. Before use, samples were quickly thawed, diluted 1:100 in brucella broth (BBL Microbiology Systems), and inoculated into the yolk sacs of 6-day-old embryonated leukosis-free eggs (SPAFAS, Norwich, Conn.). The eggs were incubated at 37°C for approximately 40 h, and the organisms were harvested by separating the yolk sac contents from the remainder of the egg and used immediately. The challenge dose was determined by plate counts. Cultures were plated on brucella broth with 1.7% agar (pH 7.2), supplemented with 7% horse blood, and incubated at 42°C in Polybags (Levin Bros. Paper Co., Chicago, Ill.) with an atmosphere of 85% nitrogen, 10% CO2, and 5% O2.

Broth cultures were also inoculated from the liquid nitrogen stock. T flasks (Falcon Labware) containing 6 ml of brucella agar with 7% sheep blood were overlaid with 5.5 ml of brucella broth supplemented with 0.04% cysteine and 0.25% serine. The inoculum was approxi-mately 103 C. jejuni organisms in 0.5 ml of brucella broth. Flasks were incubated for 18 h at 42°C, and the overlay broth was harvested and used immediately. The challenge dose was also determined by plate count.

RITARD procedure. All rabbits were 1-kg New Zealand White females (Dutchland Laboratories Inc., Denver, Pa.). The surgical procedure was as previously described by Spira et al. (20). The abdomen was opened surgically under general anesthesia and aseptic conditions. The cecum was ligated near the ileocecal junction with sterile umbilical tape, and a slip knot was used to gently ligate the terminal ileum immediately anterior to the mesoappendix. A 10-ml sample of Campylobacter jejuni-infected egg yolk or broth culture (1.35 ± 0.5 × 108 CFU) was injected into the mid-small bowel. The incision was closed in two layers, and the loose ends of the slip knot were brought out through the incision. Four hours after surgery, the slip knot was released, and additional sutures were placed as needed. Sham controls received uninfected medium at the time of surgery. All animals received food and water ad libitum after release of the temporary tie.

Monitoring of infection. Rabbits were observed daily for signs of diarrhea or death. Heart blood was collected daily for the initial 1 to 4 postoperative days from 30 consecutive rabbits and was processed in the routine manner for complete blood count, electrolytes, blood urea nitrogen, creatinine, and liver functions (serum glutamic oxalacetic transaminase and serum glutamic pyruvic transaminase). Seventeen of the 30 animals were randomly selected for arterial blood gas analyses. Blood was also cultured for the presence of viable organisms in tryptic soy broth (BACTEC. Johnston Laboratories, Inc., Cockeysville, Md.), which was incubated at 37°C and subcultured on days 5, 10, and 15 by using brucella agar with 7% horse blood.

Rectal swabs were obtained daily until rabbits died or until the end of the 2-week observation period. These swabs were plated on brucella agar with 7% horse blood and Butzler antibiotic supplement (Ox-oid).

Antibody response. Whole cell antigens for enzyme-linked immunosorbent assay studies were prepared by growing C. jejuni isolates as described above. Cells were harvested with cold phosphate-buffered saline (pH 7.2) and washed twice with cold phosphate-buffered saline before suspension in cold distilled water. Each preparation was adjusted to a protein concentration of 1.0 mg/ml, as determined by the method of Lowry et al. (14), using bovine serum albumin fraction V as the standard. Samples of each antigen were maintained frozen at −70°C. Antigen coating, serum, anti-rabbit serum, alkaline phosphatase-conjugate dilutions, and substrate were prepared and added as described by Dasch et al. (5) in 100-μl volumes per well. Immunoglobulin G (IgG) fractions of goat anti-rabbit IgG (H and L chain), goat anti-rabbit (IgM, μ-chain specific), and rabbit anti-goat immunoglobulin sera were purchased from Cappel Laboratories, Cochranville, Pa. Incubation times for serum, goat antiserum, and conjugate were 2 h at 37°C. Substrate (p-nitrophenyl phosphate: Sigma Chemical Co., St. Louis, Mo.) was allowed to react for 30 min at 37°C. Absorbance was read at 405 nm in a Titertek Multiskan spectophotometer (Flow Laboratories, Inc., Rockville, Md.). The enzyme-linked immunosor-bent assay end-point titer was defined as the reciprocal of the dilution of serum eliciting an optical density at 405 nm of 0.35 above controls without rabbit serum.

Pathological examinations. Complete postmortem examinations were done on eight infected and two control animals. Five of the infected rabbits were sacrificed 24 h after onset of diarrhea, and three rabbits were sacrificed after diarrhea of 3 to 5 days duration. Animals were sacrificed by intracardiac administration of T-61 Euthanasia, and the intestines were removed immediately. Lung, liver, spleen, kidney, and adrenal glands were also processed. Specimens were placed in Karnovsky’s fixative for 1 to 3 days. Representative sections were taken from each specimen and stained with hematoxylin and eosin. Positive sections were further examined by using Warthin-Starry stain for visualization of organisms.

Statistical analysis. All statistical analyses were done with the Fisher exact test.

RESULTS

Postchallenge diarrhea. Fifty-five rabbits received either 39-3 RLS or HC strain of C. jejuni, and 16 were inoculated with egg yolk only. Diarrhea occurred in 34 of the challenged ani-mals, and 29 died, resulting in an overall attack rate of 62% and a mortality of 53% (Table 1). All animals receiving egg yolk, including controls, experienced approximately 24 h of loose stool after surgery. Animals fed broth cultures also experienced initial loose stool, but less severely. Normal pellet stools were observed in all controls and in most experimental animals on day 1 post-inoculation. Onset of diarrhea occurred after this period. Diarrhea in infected animals was remarkable in its consistent mucous content. All control rabbits had normal pellet stools from day 1 to the end of the 2-week observation period.

The attack rates for strains 39-3 RLS and HC grown in egg were 52 and 81%, respectively. This difference is minimally significant statistically (P = 0.049). The mean incubation period

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and duration of diarrhea were similar for strains grown in egg medium (Table 1).

Rectal swabs. Rectal swabs were negative for C. jejuni before challenge. All swabs from infected animals were positive at 24 h and remained positive throughout the 2 weeks that they were sampled, except for two rabbits which had occasional negative cultures. All rectal swabs from control animals were negative for C. jejuni.

Deaths. Twenty-nine of the 55 animals (53%) receiving C. jejuni died, but none of the 16 rabbits inoculated with sterile egg yolk died during the 2-week observation period ($P = 0.001$) (Table 1). There was no significant difference in mortality between strain HC (69%) and strain 39-3 RLS (41%) ($P = 0.07$). All deaths were preceded by diarrhea, and only 5 of 34 rabbits experiencing diarrhea recovered. There were no deaths caused by cardiac puncture based on examination of heart and pericardium postmortem.

Egg versus broth cultures. After the model had been established by using Campylobacter organisms grown in embryonated eggs, we then compared this preparation with cultures grown in broth media to determine whether the yolk provided an unknown nutrient necessary for pathogenesis. Ten rabbits received broth-grown cultures, and 16 received cultures grown in egg (Table 1). There was no significant difference in attack rate ($P = 0.23$) or mortality between these

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Total no. of rabbits</th>
<th>No. of rabbits with diarrhea</th>
<th>No. of rabbits which died</th>
<th>Incubation period (mean days ± SD)</th>
<th>Duration of diarrhea (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>39-3 RLS</td>
<td>Egg</td>
<td>29</td>
<td>15 (52%)</td>
<td>12 (41%)</td>
<td>1-5 (2.73 ± 1.39)</td>
<td>1-5 (2.27 ± 1.1)</td>
</tr>
<tr>
<td>HC</td>
<td>Egg</td>
<td>16</td>
<td>13 (81%)</td>
<td>11 (69%)</td>
<td>1-4 (2.15 ± 1.14)</td>
<td>1-4 (2.62 ± 1.12)</td>
</tr>
<tr>
<td>HC</td>
<td>Broth</td>
<td>10</td>
<td>6 (60%)</td>
<td>6 (60%)</td>
<td>2-5 (4.17 ± 0.98)</td>
<td>2-6 (4.83 ± 1.17)</td>
</tr>
</tbody>
</table>

FIG. 1. Serum antibody responses as measured with a whole-cell indirect enzyme-linked immunosorbent assay. Results are shown as reciprocal titers. n, Number of rabbits sampled at each time period.
two groups. However, the incubation period and the duration of diarrhea were prolonged by 2 days in the broth group.

**Bacteremia.** Bacteremia with *C. jejuni* was seen in 26 of 27 (96.3%) rabbits sampled 24 h after challenge, but it diminished in incidence during the following 3 days. Thirteen of 24 (54.2%) were positive at 48 h, 10 of 21 (47.6%) were positive at 72 h, and 5 of 19 (26.3%) were positive at 96 h. All blood cultures on control rabbits were negative.

**Blood analysis.** Forty-two blood samples were obtained from 30 rabbits. Complete blood counts, including platelet counts, were unremarkable. Liver function tests were likewise unaffected, except for nonspecific changes in four animals near death. Serum creatinines were minimally elevated only in those rabbits with extreme azotemia.

The major blood abnormalities occurred in blood urea nitrogen and pH. If azotemia is conservatively defined as a blood urea nitrogen level greater than 50 (normal = 17.6 ± 4.36), then 6 of 9 (66.7%) rabbits were azotemic on the day of death, compared with 3 of 33 (9.1%) sampled 1 to 3 days before death (*P* = 0.001). Similarly, if acidosis is defined as a pH less than 7.26 or a serum CO$_2$ level less than 10 (normal = 22.8 ± 3.2), 6 of 9 (66.7%) were acidotic on the day of the death, compared with 2 of 33 (6.1%) on a previous day (*P* = 0.0003).

**Antibody response.** Mean serum IgM and IgG antibody responses through the 28-day course of study are shown in Fig. 1. IgM antibody responses were increased—though this is not apparent in Fig. 2 due to the scale of the graph—in 4 of 5 rabbits at day 7 (range, 1:168 to 1:631) and in 10 of 12 rabbits at day 14 (range, 1:133 to 1:251). By day 28, IgM antibody titers had returned to preinfection levels. Early serum IgG antibody responses were observed in infected rabbits, and mean titers progressively increased through the course of the study. The mean IgG titer rose from 1:198 preinfection to 1:9,087 by day 28. The broad range of IgG titers observed on each of the 3 days postinfection is appropriate for an outbred pool of animals.

**Pathology.** Abnormal histology was present in seven of eight infected rabbits examined. Lesions ranged from mild inflammatory infiltrates to frank epithelial necrosis (four of eight rabbits; Fig. 2 and 3). Such lesions were not seen in the two control animals studied. Lesions in infected animals were restricted to the terminal ileum.
FIG. 3. Proximal colon from infected rabbit. (A) Eroded mucosa, bare of superficial epithelium. The lumen (L) contains protein exudate, cellular debris, and polymorphonuclear leukocytes. The lamina propria is infiltrated with inflammatory cells (mostly polymorphonuclear leukocytes) (B). The remaining intact crypt epithelium (C) is intensely basophilic. (D) Muscle layer. Bar, 10 μm.

the unligated portion of the cecum, and the colon. Lesions seen in the cecum were often diffuse and more severe than those in other affected areas. Ileal and colonic lesions were usually multifocal and microscopic. Goblet cell hyperplasia was noted in six rabbits (Fig. 4). This consisted of loss of mucus compared with normal goblet cells (Fig. 5) and signs of intense proliferative activity such as increased mitotic figures, architectural disarray, and increased nuclear material. Organisms were not demonstrated within the lesions by the Warthin-Starry stain.

Sections of liver, lung, kidney, and adrenal glands from infected animals were unremarkable, as were those from control rabbits.

DISCUSSION

Temporary ligation of the rabbit ileum, followed by proximal inoculation of live organisms, permitted C. jejuni to establish an infection in rabbits which is similar in many ways to that seen in humans (1, 3, 4, 6). This experimental infection was characterized by an incubation period of 1 to 5 days, followed by mucous diarrhea in 64% and death in 53% of rabbits challenged. Associated findings in infected rabbits included azotemia, acidosis, and abnormal intestinal histopathology. Transient bacteremias were noted in all animals, even if no other symptoms were observed.

Our findings are consistent with those of Fitzgeorge et al. (10), who fed C. jejuni to young rhesus monkeys. This resulted in mild diarrhea in six of eight animals. Bacteremia of 3 days duration was noted in three of six animals sampled, one of which remained asymptomatic. The illness was less severe than in our rabbits, and no mortality or intestinal histopathology was found.

The virulence mechanisms of C. jejuni responsible for the infectious processes initiated with the RITARD procedure cannot be determined from the present study. However, it appears that microbial activities occurring at the intestinal level are more significant than extraintestinal invasion. Transient bacteremias after oral inoculation have been documented in chicks (18), mice (2), and rhesus monkeys (10). Bacteremia has also been described in human enteritis (13). These bacteremias, as in our model, were not necessarily associated with clinical evidence of disease. At least one blood sample from all rabbits cultured after challenged grew C. jejuni, but only 64% eventually developed diarrhea.
FIG. 4. Hyperplastic colon from infected rabbit. Note that the areas corresponding to the clear spaces in Fig. 5 are highly cellular, disorganized, and intensely basophilic (A), an effect resulting from epithelial hyperplasia. Note the mitotic figures (B). Bar, 10 μm.

FIG. 5. Control colon from uninfected rabbit. Note clear spaces (A) produced by normal amounts of mucus within the goblet cells of the control colon. Bar, 10 μm.
Diarrhea was highly associated with mortality. Eighty-five percent of the animals which developed diarrhea died within 1 to 5 days of onset. Although the definite cause of death in the rabbits was not determined, azotemia and acidosis were statistically correlated with the day of death. Severe dehydration is the most likely explanation for these findings, since other etiologies of azotemia and acidosis were eliminated, such as renal failure and significant loss of blood into the gastrointestinal tract.

Preliminary pathological results on a small sample of rabbits demonstrated inflammatory intestinal lesions which could contribute to mucous production and dehydration. These lesions were similar to those reported in human specimens. Principal findings in these clinical biopsies were edema of the lamina propria with acute inflammatory infiltrates of varying severity (1, 17), crypt abscesses, mucin depletion, and an increased mitotic index in the glandular epithelium (7, 13, 17). Although the RITARD-induced infection has a much higher incidence of bacteremia and death than has been reported in human cases, the similarity of intestinal lesions suggests that the mechanisms of virulence at the intestinal level may be similar.

The early transient elevations in serum IgM and early progressive increases in serum IgG antibody titers through the 28-day study period are consistent with an immune response to infection. These observations in the rabbit are consistent with human responses to Campylobacter infection, as recently reported by Walder and Forsgren (21), who used formalized whole bacteria in an enzyme-linked immunosorbent assay and noted both serum IgM and IgG antibody titer increases. In 6 enteritis patients with stool cultures positive for C. jejuni or Campylobacter coli, both serum IgM and IgG antibody titer increases were detected early in the course of infection, and both were significantly elevated as early as 11 days after onset of infection.

Simple colonization of the intestine with C. jejuni sp. was not sufficient for production of disease symptoms. All rabbits challenged shed the organisms for the 2-week study period. Colonization without illness has been reported in other animal models (2, 16, 18), including neonatal rabbits (8). The RITARD procedure, however, may enable sufficient numbers of organisms to colonize particular ecological niches, thus favoring subsequent activities essential for disease.

The RITARD procedure appears to provide an excellent model of Campylobacter enteritis for the study of pathophysiological mechanisms. In addition, because the rabbit is immunologically well characterized, this model is applicable to investigation of host immune responses in Campylobacter infection.