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Experimental *Yersinia enterocolitica* Enteritis in Rabbits

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Young rabbits weighing 500 to 800 g were inoculated orogastrically with clinical isolates of *Yersinia enterocolitica* (serotype O:3; enterotoxigenic; HeLa cell invasive) at a dose of 1.4×10^{10} bacteria suspended in 10% sodium bicarbonate solution. Diarrhea developed in 41 (87%) of 47 rabbits, with a mean ± standard deviation onset at 5.4 ± 2.4 days. The attack rate and onset of diarrhea were correlated with inoculum size. The 50% infectious dose was 2.9×10^8 bacteria. Bacterial colonization occurred in almost all rabbits, regardless of inoculum size. Seroconversion was demonstrated in 30 (71%) of 42 rabbits with or without diarrhea. Histopathological alterations were present in the jejuna, ileà, and colons of rabbits with diarrhea; the most pronounced changes were generally noted in the ilea. Crypt abscesses localized at the depth of the intestinal glands were observed consistently and were composed of a bacterial nidus admixed with and enveloped by inflammatory cells comprised of eosinophils, neutrophils, and mononuclear cells. Rabbits inoculated with a raw fish isolate of *Y. enterocolitica* (serotype O:6,30; non-enterotoxigenic; HeLa cell noninvasive) did not exhibit infection clinically, bacteriologically, or pathologically.

Yersinia enterocolitica is recognized as an important cause of gastroenteritis in children (2, 7, 12, 16, 31). In a prospective study conducted in Montreal, *Y. enterocolitica* was isolated from the stools of 181 (2.8%) of 6,364 children with diarrhea over a 15-month period (16). The frequency was only slightly less than the frequencies for *Salmonella* and *Campylobacter jejuni* and considerably greater than the frequency for *Shigella* (20). *Y. enterocolitica* has also been recovered frequently from animals (25, 26), water supplies (11, 13), raw milk (24), and foods (10).

Recent studies on the potential pathogenic properties of *Y. enterocolitica* as an enteric pathogen have demonstrated production of heat-stable enterotoxin (3, 18, 23), penetration of epithelial cells (14, 15, 21, 28, 29), and production of keratoconjunctivitis in guinea pigs (Séreny test) (3, 8). Although these properties are most frequently associated with clinical isolates, some environmental strains also produce enterotoxin or penetrate HeLa cells or both (14, 19, 21, 29). However, the public health significance of these environmental isolates remains uncertain. A suitable animal model needs to be established to study (i) the pathogenesis of *Y. enterocolitica* gastroenteritis, (ii) the relative importance of each of the three pathogenic properties in vivo, and (iii) the clinical significance of environmental isolates that possess one or more of the pathogenic properties.

To parallel the events in human infections, an animal model suitable for our purpose must be developed, in which diarrhea is produced after an orogastric inoculation of strains isolated from patients with diarrhea. A number of experimental models of *Y. enterocolitica* infections have been described, but they have several shortcomings. Alsonso et al. (1) described a systemic infection induced in Swiss mice with a *Y. enterocolitica* strain of serotype O:3, biotype 4, phage type VIII (the most common type isolated from human infections in Europe), but the animals had to be infected by the intravenous rather than the oral route. Ricciardi et al. (22) used mice (Porton white strain) only to establish the duration of fecal excretion of intraperitoneally introduced strains and the resistance of the mice to subsequent challenge with a lethal dose of virulent *Y. enterocolitica*. Carter (6) described a model in pathogen-free CD-1 mice fed *Y. enterocolitica* WA strain; however, clinical signs and symptoms were not described. In the rabbit model developed by Une (27), strains considered pathogenic to humans based on epidemiological data produced diarrhea and histopathological changes of enterocolitis; however, the animals were inoculated directly into the duodenal lumen through the serosa under laparotomy, which could introduce the undesirable complications of peritonitis and systemic infections. Experimental infections of monkeys (17) would be too costly.

The purpose of the present study was to develop an animal model for *Y. enterocolitica* en-

teritis in which diarrhea could be produced consistently by pathogenic strains after orogastric challenge. Rabbits were selected for these studies because of their known susceptibility to diarrhea after intraduodenal infection (27). In this preliminary work we used only *Y. enterocolitica* strains of serotype O:3 isolated from children with diarrhea as pathogenic strains and a strain of serotype O:6,30 isolated from raw fish as a nonpathogenic control.

MATERIALS AND METHODS

Bacterial strains. Three strains of *Y. enterocolitica* were employed. Two strains, MCH-628 and MCH-700, were isolated from patients with diarrhea; both are serotype O:3, enterotoxigenic, HeLa cell invasive, and Séreny negative. Strain 1193 (kindly provided by S. Toma, National Reference Centre for *Yersinia*, Laboratory Service Branch, Ontario Ministry of Health, Toronto, Ontario, Canada) was originally isolated from raw fish and is serotype O:6,30, non-enterotoxigenic, HeLa cell negative, and Séreny negative. The organisms were suspended in brain heart infusion broth (Difco Laboratories) with 20% glycerol and stored at −70°C.

Animals. New Zealand white rabbits weighing 0.5 to 0.8 kg were obtained locally (Canadian Breeding Farm Laboratories Ltd., St. Constant, Quebec, Canada) and housed in groups of two to three per cage. They were observed for 1 day to ensure the absence of diarrhea. Animals were fasted for 24 h before infection.

Preparation of inoculum. Bacteria were grown on sheep blood agar plates at room temperature for 1 day and harvested into a 10% $NaHCO_3$ solution. The turbidity of the suspension was adjusted to an optical density of 1.0 by using a Spectronic 20 spectrophotometer (Bausch & Lomb Inc.). Bacterial concentration was 1.4×10^9 colony-forming units (CFU) per ml. The suspension was diluted with 10% $NaHCO_3$ when a lower concentration of bacteria was desired.

Infection of rabbits. After body weights were measured and rectal swabs were taken for culture, animals were anesthetized intramuscularly with ketamine hydrochloride (40 mg/kg); 10 ml of $NaHCO_3$ solution containing 1.4×10^9 CFU/ml was administered through a feeding tube (size, 5 french) passed into the stomach by the oral route. The number of bacteria thus inoculated into each rabbit was 1.4×10^{10} CFU. When lesser inoculum sizes were desired, the bacterial suspension was diluted with 10% $NaHCO_3$.

Experimental design. After inoculation, animals were weighed and observed daily. Rabbits were considered to have diarrhea when feces were semisolid and their perinea or hind legs were wet and soiled. Colonization of inoculated organisms in the intestines was followed by culturing rectal swabs taken daily during the first 3 days after challenge and every other day thereafter. Blood samples were obtained at various times after inoculation to examine serological response. Animals were sacrificed with an overdose of sodium pentobarbital by intracardiac injection. Pieces of livers and spleens were taken for bacteriological examinations, and jejuna, ilea, and colons were taken for histological examinations. Intestinal contents were also cultured.

Bacteriological examination. Rectal swabs were plated onto MacConkey agar, which was then incubated at 35°C for 1 day. The number of colonies resembling *Y. enterocolitica* colonies was estimated, and representative colonies were identified by biochemical reactions. Livers and spleens were weighed and homogenized in 2 ml of phosphate-buffered saline; 0.1 and 0.001 ml of the homogenate were plated onto MacConkey agar for viable counts. A loopful of intestinal contents was also plated onto MacConkey agar for semiquantitative culture.

Serology. Agglutination antibody was determined by microtiter titration, using heat-killed suspensions of challenge organisms as well as the reference strains (*Y. enterocolitica* I.P. 134 for serotype O:3 and I.P. 102 for serotype O:6,30) that are used at the National Reference Centre for *Yersinia* for the serological confirmation of *Y. enterocolitica* infections. Bacteria were grown on Trypticase soy agar at room temperature for 48 h, harvested into phosphate-buffered saline, washed three times in buffer, and boiled for 2.5 h. After cooling, the turbidity was adjusted to a density equivalent to McFarlane tube no. 1, and the suspension was used as the source of antigen. The lowest dilution of sera tested was 1:20.

Histological examination. Sections of tissues were fixed with 10% buffered formalin, dehydrated in graded alcohols, embedded in paraffin, sectioned, and stained with hematoxylin-phloxine-saffron, Giemsa solution, and Gram stain.

Enterotoxin assay. Diarrheic animals were sacrificed, and the watery intestinal contents were centrifuged at 10,000 × *g* for 10 min; the supernatant fluid was assayed for enterotoxigenic activity in suckling mice as described previously (18).

RESULTS

Clinical. In five separate experiments, 47 rabbits were inoculated with 1.4×10^{10} CFU of two clinical isolates of *Y. enterocolitica* (Table 1). Diarrhea developed in 20 (87%) of 23 and 21 (88%) of 24 rabbits inoculated with strains MCH-628 and MCH-700, respectively. The attack rate was markedly consistent in each experiment and

TABLE 1. *Frequency of diarrhea in rabbits inoculated orogastrically with Y. enterocolitica*[a]

Strain	No. with diarrhea/ no. inoculated
MCH-628	20/23 (87)[b]
MCH-700	21/24 (88)
1193	2/19 (11)
$NaHCO_3$ alone[c]	1/9 (11)

[a] Rabbits weighing 0.5 to 0.8 kg were inoculated orogastrically with 10 ml of 10% $NaHCO_3$ containing 1.4×10^{10} bacteria and observed for 15 days.

[b] Numbers in parentheses are percentages.

[c] A 10-ml amount of 10% $NaHCO_3$ with no bacteria.

had a range of 78 to 93%. The mean ± standard deviation day of onset of diarrhea was day 5.4 ± 2.4. The duration of diarrhea could not be determined since many rabbits were sacrificed for bacteriological and pathological examinations before the cessation of diarrhea. Frequently, rabbits had had diarrhea for 5 to 6 days before sacrifice. A total of 14 (30%) of 47 rabbits inoculated died within 8.5 ± 2.2 days (mean ± standard, deviation) after inoculation. All 14 had diarrhea. The mortality rate should be the minimum estimate since several more rabbits would probably have died if they had not been sacrificed for histological examinations.

Rabbits were also inoculated with *Y. enterocolitica* 1193, a strain originally isolated from raw fish (Table 1). Only 2 of 19 rabbits had very mild diarrhea (only small areas of the perinea were wet). The frequency of diarrhea in the rabbits inoculated with strain 1193 was no different than that observed in rabbits given sodium bicarbonate solution only. Furthermore, rectal swabs obtained from the diarrheic rabbits did not grow *Yersinia*.

The rate of weight gain by rabbits inoculated with the clinical isolates was considerably lower than that in control groups (Fig. 1). The average weight of rabbits (10 to 13 in each group) inoculated with strain 1193 or fed $NaHCO_3$ alone doubled in 11 days, whereas the rabbits inoculated with the clinical isolates gained less than 20% of their pre-inoculation weight during the same period.

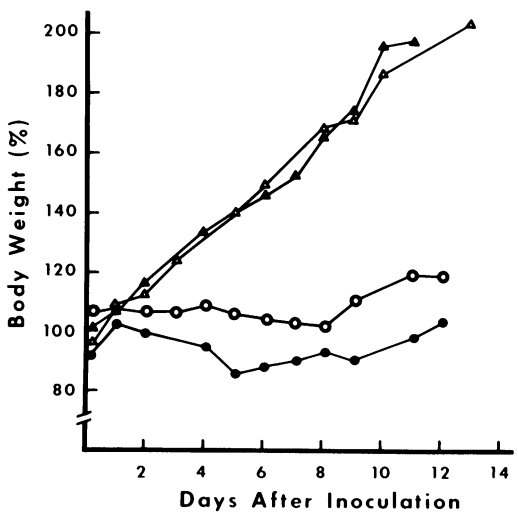

FIG. 1. *Changes in the body weights of rabbits inoculated with Y. enterocolitica. Rabbits were inoculated orogastrically with 10 ml of 10% $NaHCO_3$ containing 1.4 × 10^{10} bacteria. Symbols: ○, strain MCH-628; ●, strain MCH-700; ▲, strain 1193. Control rabbits (△) received 10 ml of the $NaHCO_3$ solution alone. The weights of the rabbits on the day before inoculation were taken as 100%.*

Effect of inoculum size. Seven groups of rabbits were inoculated with *Y. enterocolitica* MCH-700 in doses ranging from 1.4 × 10^4 to 1.4 × 10^{10} CFU (Table 2). The attack rate, as well as the length of the incubation period, varied significantly with inoculum size. The 50% infective dose (the number of bacteria that caused diarrhea in 50% of rabbits inoculated) was 2.9 × 10^8 bacteria. There was a statistically significant correlation between inoculum size and incubation period (Fig. 2) ($P < 0.01$).

Bacteriology. All rabbits challenged with strains MCH-628 and MCH-700 excreted the organisms in their stools throughout the experimental period, which varied from 2 to 3 weeks. Stool cultures usually became positive for *Yersinia* within 1 to 2 days after inoculation, and bacterial excretion continued with or without diarrhea, although the concentrations of the bacteria in the stools, as estimated from the number of small lactose-negative colonies on MacConkey agar, were higher in rabbits with diarrhea. It was often noted that the coliform bacteria that grew from the stools initially disappeared gradually as the number of *Yersinia* colonies increased in diarrheic animals. The frequency of colonization was not influenced by inoculum size; 45 of 46 rabbits challenged with various inoculum sizes (Table 2) were colonized with the challenge organism, although diarrhea developed in only 26. An epidemiological study of *Y. enterocolitica* gastroenteritis in humans revealed that 50% (10 of 20) of the household contacts who were infected were asymptomatic (16). Clinical outcome, but not colonization, may be influenced by inoculum size in human infections as well. *Y. enterocolitica* 1193, on the other hand, could not be recovered in the stools, even though five rectal swabs were obtained from each of 10 rabbits during the first 2.5 days after the challenge. Rectal swabs obtained thereafter were also negative. Of the 10 rabbits, 5 were sacrificed between days 3 and 13 post-inoculation, and the intestinal contents of the colons, ilea, and jejuna were cultured; all were negative.

The systemic dissemination of the organism was assessed by liver and spleen cultures obtained at the time of sacrifice. The cultures of livers or spleens or both were positive in five of eight rabbits inoculated with 1.4 × 10^{10} CFU of strain MCH-700. The number of organisms recovered ranged from 3 × 10^2 to 5 × 10^6 CFU/g. No organisms were recovered from these organs of five rabbits inoculated with strain 1193.

Serology. Sera were obtained from 42 rabbits inoculated with *Y. enterocolitica* MCH-700 and tested for antibody to the challenge organisms

TABLE 2. *Effect of inoculum size on the frequency of diarrhea in rabbits inoculated with Y. enterocolitica MCH-700*

Inoculum size[a] (CFU)	No. of animals inoculated	No. with diarrhea	Incubation[b] period (days)
1.4×10^{10}	9	8 (89)[c]	5.1
4.2×10^{9}	6	5 (83)	6.2
1.4×10^{9}	6	4 (67)	9.8
4.2×10^{8}	6	4 (67)	7.0
1.4×10^{8}	9	4 (44)	10.8
1.4×10^{6}	5	1 (20)	12.0[d]
1.4×10^{4}	5	0 (0)	

[a] Rabbits were inoculated orogastrically with 10 ml of 10% $NaHCO_3$ containing varying numbers of bacteria.

[b] Mean number of days before onset of diarrhea.

[c] Numbers in parentheses are percentages.

[d] Only one rabbit with diarrhea.

as well as the reference strain (*Y. enterocolitica* I.P. 134). All sera obtained before inoculation showed a titer of less than 20. A total of 30 rabbits (71%) showed a fourfold or greater increase in antibody titer in sera obtained 12 to 16 days after inoculation; the geometric mean titer was 62. The antibody titers determined against the challenge and reference strains did not differ by more than one dilution. Seroconversion was not demonstrable in 3 of 19 rabbits with diarrhea and in 9 of 23 without diarrhea, but the difference was not significant.

None of four rabbits inoculated with strain 1193 demonstrated any serological response; the titers of sera taken at 14 days post-inoculation remained at less than 20.

Enterotoxigenic activity of intestinal contents. Intestinal contents of the colons, ilea, and jejuna were obtained from 14 diarrheic rabbits challenged with *Y. enterocolitica* MCH-628, and supernatant fluids were assayed for enterotoxigenic activity in suckling mice. All were negative.

Pathology. Representative experimental animals inoculated with the clinical isolates of *Y. enterocolitica* were sacrificed at periodic intervals from day 1 to day 9 after the onset of diarrhea. Several sections of jejunum, ileum, and colon were examined. Histopathological alterations were present at each site in all animals; the most pronounced changes were generally noted in ilea.

The early lesion consisted of bacterial invasion of glands, with extensions into the depths of the glands to form crypt abscesses. The latter were composed of a somewhat spherical nidus of gram-negative coccobacilli admixed with and surrounded by inflammatory cells (Fig. 3A). The adjacent crypt epithelium was often severely degenerated and focally necrotic. The inflammatory component was composed of a mixture of eosinophils, neutrophils, lymphocytes, and macrophages (eosinophils were confirmed by Giemsa stain) (Fig. 3B). With time, contiguous individual crypt abscesses became confluent as the inflammatory process spread laterally in the lamina propria. Minute areas of mucosal ulceration were observed overlying an occasional crypt abscess, especially in sites rich in lymphoid tissue. The latter was diffusely hyperplastic and demonstrated a marked immunoblastic response. In addition, many submucosal lymphoid nodules revealed necrosis of follicular centers. Neither pseudomembrane development nor epithelioid granuloma formation was seen. Only infrequently were inflammatory aggregates observed penetrating the muscularis mucosa into the submucosa; in no instance did the process involve the muscularis propria or serosa.

Rabbits inoculated with *Y. enterocolitica* 1193 or given sodium bicarbonate alone (including those with mild diarrhea [Table 1]) were sacrificed on days 3 to 13 post-inoculation. No pathological findings were observed.

DISCUSSION

This study demonstrated that young rabbits can be used successfully as an experimental model for *Y. enterocolitica* enteritis. Diarrhea was produced in the animals reliably and reproducibly by orogastric inoculation of clinical iso-

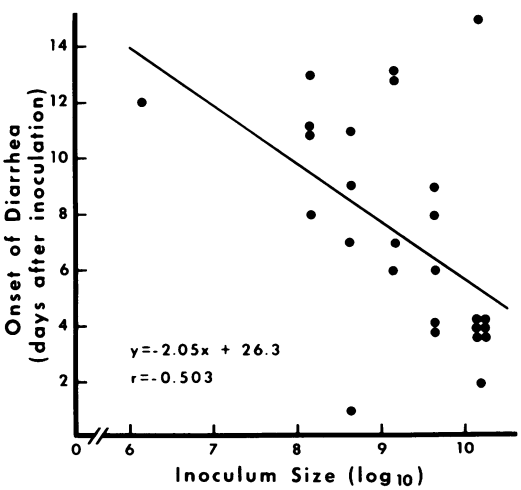

FIG. 2. *Relationship of inoculum size to period between inoculation and onset of diarrhea in rabbits infected with Y. enterocolitica MCH-700. Rabbits were inoculated orogastrically with 10 ml of 10% $NaHCO_3$ containing varying numbers of bacteria.*

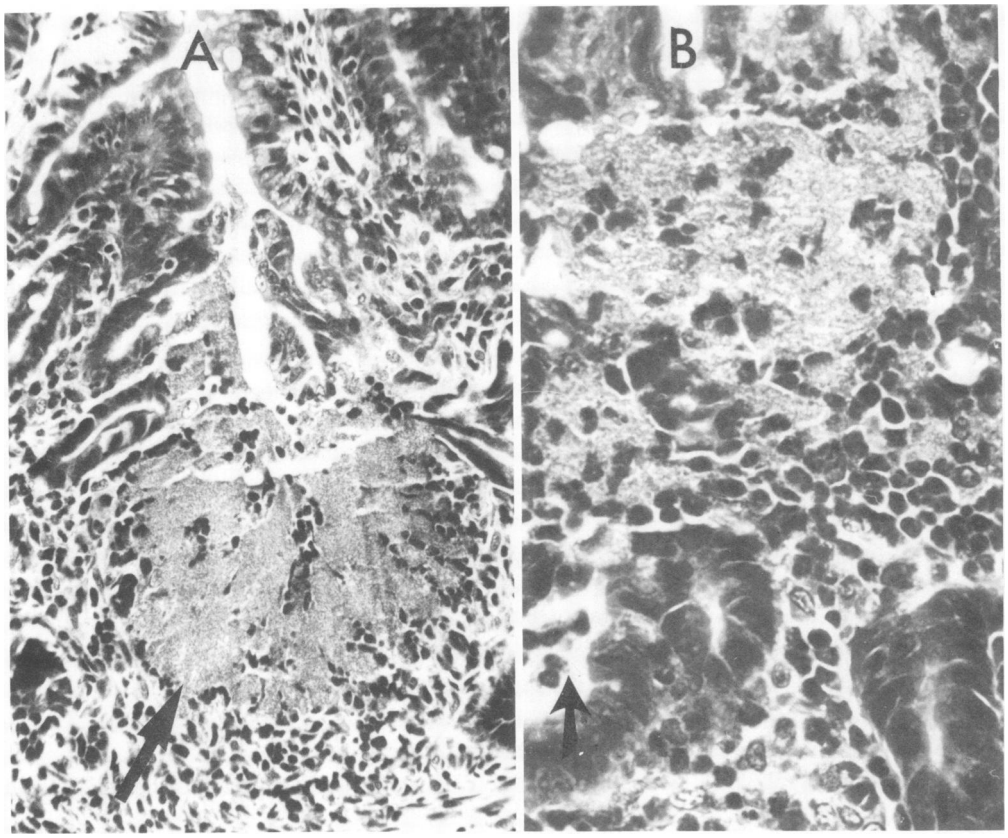

FIG. 3. *Light micrographs of sections of ilea taken from rabbits inoculated with Y. enterocolitica MCH-700 (hematoxylin-phloxine-saffron stains). (A) Bacterial nidus (arrow) in depth of intestinal crypt. Note inflammatory response around the nidus. ×250. (B) Bacteria mixed with inflammatory component around and within (arrow) intestinal glands. ×400.*

lates suspended in $NaHCO_3$ solution. The attack rate and the onset of diarrhea were correlated with inoculum size. Diarrhea, asymptomatic colonization, a long period of shedding, serological response with or without diarrhea, and an invasion of intestinal mucosa by the organism were the features of the experimental infection that have also been observed in human infections (2, 4, 16). These features were completely absent in rabbits challenged with a strain of *Y. enterocolitica* isolated from raw fish.

The agglutinating antibody titers in the infected rabbits were lower than those reported in humans (16) and in other experimental models (6, 17, 27). The difference may be due in part to the age of the rabbits employed in the present study. Diminished antibody responses in young infants infected with *Y. enterocolitica* have been reported previously (16).

The clinical isolates used in this study are serotype O:3, elaborate enterotoxin in culture supernatant fluid, and are capable of penetrating HeLa cells. The histopathological changes induced in the rabbits by the clinical isolates clearly indicated that the ability of the organism to penetrate the intestinal mucosa played an essential role in the pathogenesis of experimental enteritis. The correlation between HeLa cell penetration and virulence has been described previously (27, 28). The role of enterotoxin, however, could not be ascertained in the present study. Our attempt to demonstrate enterotoxin in the watery intestinal contents of diarrheic rabbits was not successful. Production of heat-stable enterotoxin in vivo by *Escherichia coli* has been demonstrated in experimentally infected animals (30). Enterotoxin was produced not only by clinical isolates, but also by environmental isolates of *Y. enterocolitica* (19). Furthermore, the enterotoxin was detectable in culture filtrates only when the organisms were grown at low temperatures (<30°C) (18). These findings are not consistent with the hypothesis that the enterotoxin of *Y. enterocolitica* plays

an important role in the pathogenesis of diarrheal diseases. Mutant strains of *Shigella dysenteriae* that elaborate enterotoxin but, unlike their wild-type parents, are not able to invade the bowel, did not induce overt diarrhea in monkeys (9).

We do not know how critical the size of the rabbits or the choice of the $NaHCO_3$ solution is to the success of this animal model. In one experiment at the beginning of this study, five rabbits weighing an average of 1 kg were inoculated with strain MCH-628. Diarrhea was produced in only two (40%). In the very next experiment, the attack rate increased to 93% (13 of 14) when 600-g rabbits were used. However, the effect of animal size on the attack rate was not examined further. It should be noted that diarrheal diseases associated with *Y. enterocolitica* have been observed most frequently in children less than 3 years old (16). In another experiment, attack rates were compared in rabbits challenged with 1.4×10^{10} CFU of strain MCH-700 suspended in $NaHCO_3$ solution or phosphate-buffered saline. No difference was observed. However, we did not examine the 50% infective dose under these different experimental conditions. Administration of sodium bicarbonate has been used successfully in experimental *E. coli* diarrhea in rabbits (5).

The condition of the bacterial strains administered appears to be most critical to the success of this animal model. *Y. enterocolitica* strain MCH-628 was isolated from a patient with diarrhea in August 1977 and was immediately frozen at −70°C. Animal experiments performed in August 1978 showed an attack rate of 87% (13 of 14 and 7 of 9), which gradually decreased to 40% (4 of 10) during a period of 6 months. The strain remained enterotoxigenic and HeLa cell invasive and was capable of colonizing rabbit intestines, but was no longer capable of producing diarrhea and histopathological alterations of the intestines as before. Animal experiments were then conducted with a fresh clinical isolate (MCH-700), which produced diarrhea in about 80 to 90% (8 of 9, 9 of 10, and 4 of 5 in three experiments) of the rabbits inoculated. Attenuation of *Y. enterocolitica* during storage has been noted by others (6).

The pathological alterations noted in the rabbits with diarrhea due to the clinical isolates were similar to those described by Maruyama (17) and Une (27) in experimental *Y. enterocolitica* infections in monkeys and rabbits. (i) Most pronounced changes were present in the ileum, although lesions were also demonstrated in the colon and jejunum. (ii) Bacteria multiplied at the depth of the intestinal gland mixed with and surrounded by inflammatory cells (crypt abscess), followed by the spreading of the inflammatory process laterally in the lamina propria. And (iii) microscopic foci of minimal ulceration were present at the sites containing abundant lymphoid tissue (Peyer's patch), which were hyperplastic and displayed foci of necrosis confined to germinal centers. However, differences were also noted; in neither the monkeys nor the rabbit model of Une did the authors describe an infiltration of eosinophils, which in our study was a major cell type of the inflammatory component in the crypt abscesses. Mononuclear cells were the predominant type in the monkey model and in the rabbit model of Une.

In the experimental infections of *Y. enterocolitica* in mice described by Carter (6), the pathological changes were more pronounced than those described in the other animal models and those observed in our study. The early lesions in the Peyer's patches in the distal ilea characterized by a marked infiltration of neutrophils progressed rapidly to both mucosal ulceration and perforation of the bowels at sites of the submucosal lymphoid tissue, with ensuing peritonitis. In no instance did the inflammatory process extend to the muscularis propria or serosa in the other animal models or in our rabbit study. The pathological differences noted in the mouse model compared with other animal studies may be attributable in part to the differences in the *Yersinia* strains used. WA strain (serotype O:8), which was employed in the mouse model, is Séreny positive (Mors and Pai, submitted for publication), whereas the clinical isolates (serotype O:3) employed in our study are Séreny negative, although all are enterotoxigenic and are capable of penetrating HeLa cells. The *Yersinia* strains used in the monkey model and in the rabbit model of Une are serotype O:3 or O:9 or both and are without exception Séreny negative (Mors and Pai, submitted for publication). In an outbreak of abdominal illness caused by Séreny-positive, serotype O:8 *Y. enterocolitica*, abdominal pain was the predominant symptom, and appendectomies were performed in 16 of 38 ill patients (2). In contrast, in children infected with serotype O:3 (Séreny negative), which is endemic in Eastern Canada, diarrhea is the major symptom, and appendectomies are rarely necessary (7, 16).

The reproducibility of attack rate, simplicity of experimental procedures, and low initial and maintenance costs of animals confer obvious advantages to our rabbit model compared with the other models previously described. For example, only 3 of 10 orally infected monkeys developed diarrhea. Clinical symptoms were not

described in the mouse model. In the rabbit experiment of Une, the challenge organisms were inoculated directly into the duodenal lumen through the serosa under laparotomy.

We recently described five pathogenic groups of *Y. enterocolitica* based on the three potential pathogenic properties of enterotoxin production, HeLa cell penetration, and Séreny reaction (Mors and Pai, submitted for publication). In the present study, we used strains representing only two of the five groups to develop an experimental model. Studies are in progress to compare the virulence of and histopathological changes induced by each pathogenic group when this animal model is used.

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