Invasiveness of *Yersinia enterocolitica* lacking the virulence plasmid: an in-vivo study

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Summary. Rabbits were given, by the intra-gastric route, two isogenic strains of *Yersinia enterocolitica* that differed only in the presence or absence of the virulence plasmid. Clinical illness and characteristic morphological lesions of *Y. enterocolitica* infection were seen only in rabbits infected with the plasmid-bearing strain (MCH700S). Although rabbits infected with a strain lacking the plasmid (MCH700L) remained healthy, mild histological changes in the small intestine, consisting of epithelial-cell damage, dilatation of lymphatics and a slight increase in neutrophil polymorphonuclear leukocytes in lamina propria, were seen in the first 12 h after inoculation. Bacteria, which were identified as *Y. enterocolitica* by indirect fluorescent antibody staining, were seen in dilated lymphatics. These early lesions tended to abate quickly and were no longer detectable at 24 h. Strain MCH700L was recovered from the mesenteric lymph nodes in increasing numbers until 24 h after inoculation; the number then began to decrease rapidly. In contrast, the early lesions in rabbits given strain MCH700S progressed to micro-abscesses, focal destruction of villi, and ulcerations beginning 24 h after inoculation; the number of bacteria recovered from the lymph nodes continued to increase beyond 24 h after inoculation. Bacteria were also recovered from the liver and spleen. These results suggest that both plasmid-bearing and non-bearing strains of *Y. enterocolitica* are capable of penetrating the intestinal mucosa. However, the virulence plasmid is required for invading bacteria to proliferate in the host tissue and to establish infection.

Introduction

*Yersinia enterocolitica* is an important cause of gastroenteritis. Histopathological studies of human cases (Bradford *et al.*, 1974) and experimental infection in animals (Carter, 1975; Une, 1977; Pai *et al.*, 1980) have shown that invasion of the intestinal mucosa is of primary importance in pathogenesis. With the recent discovery of a virulence plasmid in *Y. enterocolitica*, attention has been focused on the mode of action of plasmid-encoded determinants in pathogenesis (Gemski *et al.*, 1980a; Zink *et al.*, 1980; Portnoy *et al.*, 1981; Portnoy and Martinez, 1985). However, a role for the virulence plasmid in tissue invasion is uncertain.

The invasiveness of *Y. enterocolitica*, as measured by the Sereny test (keratoconjunctival infection of guinea-pigs or rabbits; Sereny, 1955), requires the presence of the virulence plasmid (Zink *et al.*, 1980; Schiemann and Devenish, 1982). On the other hand, studies in tissue-culture cells showed that the ability of the organism to penetrate epithelial cells is restricted to certain serotypes or biotypes, but is not associated with the virulence plasmid (Portnoy *et al.*, 1981; Pai and deStephano, 1982; Schiemann and Devenish, 1982). In fact, strains lacking the plasmid have been reported by some investigators to be more invasive to culture cells than plasmid-bearing, isogenic strains (Vesikari *et al.*, 1981, 1983; Heesemann *et al.*, 1984). Recently, Bakour *et al.* (1985) and Robins-Browne *et al.* (1985) presented evidence that strains lacking the plasmid may also be invasive *in vivo*. In support of these findings is a report by Isberg and Falkow (1985) that a single chromosomal genetic locus from *Y. pseudotuberculosis* is sufficient to convert *Escherichia coli* K12 into an organism capable of invading HEP-2 cells. *Y. pseudotuberculosis* is an invasive pathogen that possesses a virulence plasmid closely related to that of *Y. enterocolitica* (Gemski *et al.*, 1980b; Bölin *et al.*, 1982; Portnoy and Martinez, 1985).

Recent studies from our laboratory showed that the plasmid-encoded cell-surface components of *Y.
enterocolitica act as antiphagocytic factors (Lian and Pai, 1985; Lian et al., 1987). It may be hypothesised that certain serotypes or biotypes of Y. enterocolitica are capable of invading the intestinal epithelial cells, irrespective of the presence of the virulence plasmid, but only plasmid-bearing strains can proliferate within the mucosal tissues and induce advanced histopathological changes. Invasive, but plasmid-free strains would be quickly eliminated from the tissues by phagocytosis, leaving no trace of tissue invasion when examined a day or two after oral challenge. The present study was designed to test this hypothesis. Using a rabbit model, we describe the interaction between Y. enterocolitica strains and the intestinal tissue at very early stages of infection and the ability of these strains to disseminate to lymphatics and other organs.

Materials and methods

Bacterial strains

Two isogenic strains of Y. enterocolitica serotype O3, biotype 4, MCH700S (plasmid-bearing) and MCH700L (plasmid-free), were used in the study. Strain MCH700S was originally isolated from a patient with diarrhoea; strain MCH700L was derived from it by growth on magnesium oxalate agar (Pai and deStephano, 1982). Virulence in experimental infection, invasiveness in tissue-culture cells, and plasmid profiles of the strains have been described previously (Pai and deStephano, 1982; Lian and Pai, 1985).

Infection of rabbits

The rabbit model developed in our laboratory (Pai et al., 1980) was used to evaluate the invasive capacity of the two isogenic strains. Briefly, New Zealand white rabbits, 0.4–0.7 kg, were deprived of food for 18 h and were given, by the intra-gastric route through a feeding tube, $10^{10}$ bacterial cells, harvested from an overnight culture on sheep-blood agar incubated at 25°C, and suspended in 10 ml of NaHCO₃ (10%, w/v) solution. After infection, the animals were allowed free access to water and food. Two groups of five rabbits, each infected with either strain MCH700S or strain MCH700L, were observed for 14 days to assess clinical symptoms, weight loss and bacterial shedding. Other groups of five to seven rabbits were killed (one animal at each sampling time) at various times after infection by an overdose of sodium pentobarbital; portions of the mesenteric lymph nodes, spleen, liver, Peyer’s patches, small intestine and ileal contents were removed for colony counts or histopathological examination.

Quantitative bacterial cultures

Tissue samples taken aseptically were placed in a known volume of cold 0.1 M phosphate-buffered saline (PBS, pH 7.2). Sections from the ileum (Peyer’s patches and surrounding gut tissue) were carefully removed, washed three times with cold PBS to remove residual ileal contents, weighed and then placed in a known volume of PBS. Samples were homogenised and serially diluted with PBS, and 0.1-ml samples were plated on to MacConkey agar in triplicate for viable counts. Cardiac blood was cultured on sheep-blood agar directly. All plates were incubated at 25°C for 48 h. Some representative lactose-negative colonies were confirmed as Y. enterocolitica by the API 20E system (Analytab Products, Plainview, NY, USA). To confirm the presence or absence of the virulence plasmid in recovered isolates, randomly-picked colonies were tested for autoagglutination, a character which has been shown to be plasmid-mediated (Laird and Cavanaugh, 1980).

Histopathological examination

Tissue samples for histology were fixed in formaldehyde 4% w/v in phosphate buffer. Segments of intestine were cut open longitudinally and allowed to adhere with the serosal surface on to index cards before fixation in buffered formalin. After fixation, longitudinal sections were taken and carefully embedded in paraffin wax or glycol methacrylate to provide sections perpendicular to the mucosa. Sections were stained with haematoxylin and eosin or Giemsa stain. Tissue for transmission electronmicroscopy was fixed in glutaraldehyde 2.5% w/v in cacodylate buffer (0.1 M, pH 7.4), post-fixed in osmium tetroxide 2% w/v in cacodylate buffer (0.1 M, pH 7.4) for 1 h, dehydrated through graded acetone, and embedded in Epon 812. Sections of 1 μm were stained with toluidine blue. Thin sections of selected areas were stained with uranyl acetate and lead acetate and were examined in a Hitachi H-600 electronmicroscope.

Samples used for fluorescence microscopy were fixed in Michel’s fixative (Michel et al., 1972). Fixed tissues were washed in Michel’s buffer before being snap frozen in liquid nitrogen-cooled isopentane and sectioned in a cryostat. Frozen sections were washed, fixed in absolute methanol, and stained with rabbit hyperimmune sera raised against Y. enterocolitica MCH700S or MCH700L and with fluorescein-conjugated goat anti-rabbit immunoglobulins (Miles Laboratories, Elkhart, IN, USA). Sections were examined with an epifluorescent microscope (Olympus Model BH-2).

Results

Virulence in rabbits

Diarrhoea and weight loss were seen in all of five rabbits infected with strain MCH700S, whereas the five animals infected with strain MCH700L
continued to gain weight and did not have diarrhoea during the 14-day period of observation. In MCH700S-infected rabbits, cultures of rectal swabs yielded a heavy growth of *Y. enterocolitica* throughout the observation period. The isolates were autoagglutination-positive, indicating that the virulence plasmid was maintained *in vivo*. In MCH700L-infected animals, rectal swabs were yielded a light growth of *Y. enterocolitica* only during the first 4 days after inoculation.

**Bacterial colonisation**

Colonisation of the intestine by *Y. enterocolitica* was assessed from 4 h after inoculation by quantitating both free luminal bacteria and tissue-associated bacteria (table I). Strain MCH700S was recovered in large numbers from the Peyer's patches, ileal tissue and ileal contents, and the numbers of bacteria remained steady during the 14-day experimental period. In contrast, strain MCH700L was recovered from the same sites in much lower numbers and during only the first 4 days after inoculation.

**Invasion**

The capacity of *Y. enterocolitica* strains to invade the intestinal mucosa was assessed by viable counts of mesenteric lymph nodes, spleen, liver and cardiac blood (table II). Both strains were recovered from the mesenteric lymph nodes as early as 1 h after inoculation and their numbers increased gradually until 24 h. From 24 h after inoculation, however, counts of strain MCH700L began to decrease and were too low to be detected at 96 h. In contrast, strain MCH700S was recovered from the lymph nodes in increased numbers at 48 h. Only strain

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**Table I. Recovery of *Y. enterocolitica* strains MCH700S and MCH700L from the intestinal tissues and luminal contents of rabbits after intragastric administration of $1 \times 10^{10}$ viable bacteria**

<table>
<thead>
<tr>
<th>Time after administration (h)</th>
<th>Peyer's patches</th>
<th>Ileal tissue</th>
<th>Ileal contents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCH700S</td>
<td>MCH700L</td>
<td>MCH700S</td>
</tr>
<tr>
<td>4 hours</td>
<td>5.0</td>
<td>ND</td>
<td>4.7</td>
</tr>
<tr>
<td>1 day</td>
<td>8.1</td>
<td>4.7</td>
<td>7.0</td>
</tr>
<tr>
<td>4 days</td>
<td>6.3</td>
<td>2.3</td>
<td>6.3</td>
</tr>
<tr>
<td>8 days</td>
<td>7.8</td>
<td>0</td>
<td>7.3</td>
</tr>
<tr>
<td>14 days</td>
<td>7.2</td>
<td>0</td>
<td>6.7</td>
</tr>
</tbody>
</table>

ND = not determined.

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**Table II. Recovery of *Y. enterocolitica* strains MCH700S and MCH700L from the internal organs of rabbits after intragastric administration of $1 \times 10^{10}$ viable bacteria**

<table>
<thead>
<tr>
<th>Time after administration (h)</th>
<th>Mesenteric lymph nodes</th>
<th>Spleen</th>
<th>Liver</th>
<th>Cardiac blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCH700S</td>
<td>MCH700L</td>
<td>MCH700S</td>
<td>MCH700L</td>
</tr>
<tr>
<td>1</td>
<td>2.1</td>
<td>2.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2.2</td>
<td>1.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2.9</td>
<td>2.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>2.9</td>
<td>3.6</td>
<td>1.7</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>4.0</td>
<td>4.2</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>6.3</td>
<td>1.5</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>96</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
</table>

ND = not determined.
MCH700S was isolated from spleen and liver. Cardiac blood of animals infected with either strain was sterile.

Pathology

Microscopic intestinal lesions were seen in both groups of rabbits at 1–12 h after inoculation (early lesions), but only in rabbits given strain MCH700S did the lesions increase progressively thereafter; rabbits given strain MCH700L displayed little histopathological changes at 24 h or later. The early lesions consisted of epithelial-cell degeneration (figs. 1 and 2) dilatation of lymphatics (fig. 3), and a slight increase in neutrophil polymorphonuclear leukocytes in lamina propria (fig. 4). The involved epithelial cells appeared lightly stained with disruption of the luminal border or were fragmented showing karyolysis (figs. 1 and 2). Lesions were present in the upper jejunum as early as 1 h after inoculation and were later seen in the entire small intestine, distributed randomly on the villi and on the surface of Peyer's patches. The histopathological changes, including dilated lymphatics and increase in polymorphonuclear leukocytes, tended to abate from 12 h after inoculation in rabbits given strain MCH700L. In contrast, rabbits given strain MCH700S showed further histopathological changes, beginning at 24 h after inoculation, characterized by the progression of the early lesion to the presence of bacterial clumps with formation of micro-abscesses, associated with focal destruction of villi and ulceration. These advanced lesions were most prominent in the ileum, particularly in Peyer's patches and surrounding tissues (fig. 5).

By light microscopy and electronmicroscopy, bacteria were sometimes seen associated with damaged epithelial cells and in dilated lymphatics of villi in rabbits given MCH700S and MCH700L (figs. 1, 2, and 3). With indirect fluorescent-antibody staining, brightly fluorescent yellow-green bacteria were seen, both on the epithelial lining and in lamina propria of both groups of rabbits (fig. 6). However, in rabbits infected with strain MCH700S, increasing numbers of bacteria were seen in the epithelial layers, lamina propria and Peyer's patches with time, while in rabbits infected with strain MCH700L, bacteria were rarely seen in lamina propria from 24 h after infection. Electronmicroscopy of MCH700S-infected rabbit sections at 24 h after inoculation showed micro-colonies of bacteria surrounded by polymorphonuclear leukocytes without any evidence of the bacteria being phagocytosed (fig. 7).

Sections of mesenteric lymph nodes, liver and spleen were also examined for histological changes. In rabbits infected with strain MCH700S, micro-abscesses with clumps of bacteria interspersed with inflammatory cells were seen in mesenteric lymph...
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damaged epithelial cells and in dilated lymphatics; and (iv) indirect immunofluorescent staining showed the presence of Y. enterocolitica within lamina propria. It should be noted, however, that the mucosal invasion by strain MCH700L, unlike the invasion by the plasmid-bearing strain (MCH700S), did not result in advanced inflammatory lesions in lamina propria and Peyer's patches, nor invasion beyond the mesenteric lymph nodes. Mild histopathological changes seen in rabbits infected with strain MCH700L were limited to those sections taken during the first 12–24 h after infection, and histological findings were without any abnormality after 24 h. Furthermore, occasional bacteria seen in lamina propria up to 12 h after infection were no longer detectable after 24 h. In contrast, rabbits infected with strain MCH700S showed progressive lesions with micro-abscess formation, villus destruction and ulceration, and bacteria were recovered in increasing numbers from the mesenteric lymph nodes, as well as from the liver and spleen.

Fig. 3. Dilated lymphatics containing bacteria (arrows) in lamina propria of upper jejunum of a rabbit infected with strain MCH700S, 4 h after inoculation. Glycol methacrylate section; Giemsa stain. Bar = 20 μm.

Discussion

In this study, we have presented evidence that a Y. enterocolitica strain (MCH700L) lacking the virulence plasmid is capable of penetrating the intestinal mucosa of rabbits, although the strain failed to produce clinical illness and advanced histopathological changes in the animal. This conclusion was based on the following observations: (i) a significant number of viable bacteria was recovered from the mesenteric lymph nodes; (ii) histopathological changes were seen, including epithelial-cell damage, dilatation of lymphatics and a slight increase in inflammatory cells in lamina propria; (iii) bacteria were seen associated with the nodes beginning at 24 h after infection. Little histopathological change was detected in the liver and spleen. No significant abnormality was seen at any time in the above tissues from rabbits given strain MCH700L.

Fig. 4. Mild acute inflammatory response in lamina propria as shown by presence of polymorphonuclear leukocytes (arrows) in a rabbit infected with strain MCH700L, 3 h after inoculation. Paraffin section; haematoxylin and eosin stain. Bar = 10 μm.
Fig. 5. Advanced lesions in Peyer's patches and surrounding tissues 24 h after inoculation of strain MCH700S, showing bacterial clumps (arrows) with formation of micro-abscesses, destruction of villi and ulceration. Glycol methacrylate section; Giemsa stain. Bar = 20 μm.

Fig. 6. Fluorescent antibody stain showing presence of Y. enterocolitica on epithelial surface and in lamina propria, 12 h after inoculation of strain MCH700L. Bar = 20 μm.

Fig. 7. Electronmicrograph of Peyer's patch in distal ileum 24 h after inoculation of strain MCH700S. Bacteria (arrows) are in close contact with neutrophils (N) with no evidence of being ingested. Bar = 1 μm.
The present study confirms the recent reports by Bakour et al. (1985) and Robins-Browne et al. (1985) that strains of *Y. enterocolitica* lacking the virulence plasmid invade the intestinal mucosa. Plasmid-free strains of *Y. enterocolitica* were recovered from the mesenteric lymph nodes of mice (Bakour et al., 1985) and the mesenteric lymph nodes, liver and spleen of gnotobiotic piglets (Robins-Browne et al., 1985). Furthermore, anti-*Y. enterocolitica* antibodies were detected in the sera of mice challenged orally with a plasmid-free strain (Bakour et al., 1985). The present study shows also, for the first time, that infection with a plasmid-free strain produced mild early histopathological changes in the intestinal mucosa. In other experimental infections, histological examinations were not performed until 24 h after inoculation or later (Robins-Browne et al., 1985). The histopathological changes seen in this study in rabbits infected with the plasmid-free strain would have been missed completely if the first necropsy had not been performed until 24 h after inoculation. The presence of bacteria in lamina propria would not have been easily observed at that time. Also, the use of glycol methacrylate sections (figs. 1 and 3) in our study would enhance the detection of early epithelial changes and of the presence of bacteria because of better resolution afforded by these plastic-embedded sections.

That invasiveness of the gut epithelium by *Y. enterocolitica* is not a plasmid-associated event is consistent with the in-vitro finding that plasmid-free strains were as invasive as plasmid-bearing parent strains in tissue cultures (Portnoy et al., 1981; Vesikari et al., 1981; Pai and deStephano, 1982; Schiemann and Devenish, 1982; Vesikari et al., 1983; Heesemann et al., 1984). The virulence factor(s) encoded by the plasmid may, therefore, appear to decide the fate of the organism subsequent to the mucosal invasion. The plasmid-free strain appears to be unable to proliferate in the tissue and to establish destructive loci in lamina propria and Peyer's patches. In contrast, the plasmid-bearing strain continues to multiply and forms microcolonies, causing severe inflammatory responses and tissue destruction. In these foci, bacteria were found surrounded by, or interspersed with, inflammatory cells, but there was little evidence that the bacteria were being phagocytosed. These observations support our recent finding that plasmid-encoded cell-surface components of *Y. enterocolitica* act as antiphagocytic factors (Lian and Pai, 1985; Lian et al., in press). It appears that the ability of *Y. enterocolitica* to penetrate the intestinal epithelial cells is not associated with the virulence plasmid, but only plasmid-bearing strains are capable of proliferating within host tissues and establishing infection because of their resistance to phagocytosis, which is a plasmid-associated character.

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