Effect of endotoxin on colonisation of *Campylobacter jejuni* in infant mice

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Summary. An infant mouse model has been used to investigate the colonisation of the intestine by *Campylobacter jejuni* and the effect of endotoxin (*Escherichia coli* O26:B6) on the initial stage of this process. Endotoxin injected 1 or 16 h before the bacterial challenge had no effect on the growth of campylobacters but endotoxin injected 4 to 10 h before the bacterial challenge caused a bacteriostatic effect on the growth of campylobacters which lasted for one day. The bacteriostatic effect was evident both in the small intestine and in the distal part of the intestine containing caecum and colon. The mechanism of the bacteriostatic effect of endotoxin could not be explained in the study, but is thought to be non-immunological because it developed so rapidly. Oral and parenteral iron administered as ammonium ferric citrate or iron dextran, respectively, were used in an attempt to reverse the bacteriostatic effect. High oral doses of iron (0.5 mg per animal) were effective but small doses (0.5 mg per animal) were ineffective. Parenteral iron administration had a delayed effect on the reversal of the bacteriostatic effect of endotoxin. Transferrin administered orally caused a clear bacteriostatic effect in both endotoxin pretreated and untreated mice. *Campylobacter* counts were always lower in the small intestine than in the large intestine both in control and in endotoxin pretreated mice. This indicates that the large intestine is the primary ecological niche where campylobacters colonise mice.

Introduction

During the last 10 years several animal models have been developed to mimic human campylobacter infection: these include mice (Field *et al.*, 1981; Blaser *et al.*, 1983; Kazmi *et al.*, 1984; Fauchere *et al.*, 1985; Lee *et al.*, 1986; Yrios and Balish, 1986), rabbits (Caldwell *et al.*, 1983) and chickens (Beery *et al.*, 1988; Shanker *et al.*, 1988). In experimental infections, infant animals are more susceptible than older animals to colonisation with campylobacters (Field *et al.*, 1981). Gnotobiotic animals have been colonised persistently (224 days) with *Campylobacter jejuni*, but when the animals were allowed to acquire a more complex intestinal flora, the campylobacters were gradually eliminated (Yrios and Balish, 1986). Using an adult mouse model, Blaser *et al.* (1983) noted that a large inoculum of 10^8 cells per animal was needed for colonisation. In many of the animal models, however, animals showed neither diarrhoea nor other clinical symptoms comparable with human campylobacter enteritis.

Colonisation of the intestinal tract by a patho-

genic organism is the first stage in the pathogenesis of infection. In mice, campylobacters have been shown to be mucus colonisers (Field *et al.*, 1981; Lee *et al.*, 1986). Adhesion to intestinal epithelial cells could be the mechanism for intestinal colonisation by campylobacters, but no specific adhesins indicating this to be the case have been found in these organisms. In their spiral morphology and motility characteristics, campylobacters closely resemble normal intestinal microbiota inhabiting the mucous layer (Lee *et al.*, 1986). The second stage in colonisation is the multiplication of the organism in special niches of the intestinal tract. In animal models, campylobacters colonise the intestinal tract from the stomach to the colon (Blaser *et al.*, 1983; Field *et al.*, 1984; Lee *et al.*, 1986; Yrios and Balish, 1986), the highest counts of the organism being found in the caecum and colon. Host-microbe interactions play a significant role in colonisation and a variety of mechanisms at intestinal surfaces have evolved to resist colonisation by pathogens. Immunologically, the most important mechanism is the production of IgA, which is secreted into the mucous layer. The non-immunological mechanisms important in counteracting colonisation are avail-

ability of nutrients, an indigenous mucus-associated flora, and the secretion by intestinal epithelial cells of bacteriostatic and bacteriocidal substances like lysozyme and lactoferrin (McNabb and Tomasi, 1981). The importance of secretory IgA in the prevention of colonisation by campylobacters has been shown indirectly in human and animal studies (Lane *et al.*, 1987; McSweeney *et al.*, 1987). The role of non-immunological mechanisms *in vivo* is less well known.

In the present study, the effects of systemically injected endotoxin on the colonisation kinetics of *C. jejuni* strains in infant mice were examined. Endotoxin pretreatment is known to have immediate effects on host iron metabolism, i.e., on the kinetics of the iron-binding proteins, lactoferrin, transferrin and ferritin, in the body (Weinberg, 1978; Bullen, 1981). The effects of endotoxin in many aspects resemble the acute phase effects of infection caused by gram-negative bacteria.

Materials and methods

Bacterial strains

Three *C. jejuni* strains isolated from campylobacter enteritis were used: strain 2605 from a child, strain 12650 from an adult (both infections acquired in Finland) and strain KH from an adult infected in Central Europe. The strains were subcultured several times on Brucella Blood Agar (Bacto Brucella Agar, Difco, Detroit, MI) before storage at -70°C . Before use as an inoculum each strain was passaged once in mice.

Animals

The experiments were performed by permission of the Experimental Animal Committee of the College of Veterinary Medicine. NMRI mice purchased from Orion, Espoo, Finland, were used to establish a breeding colony. The animals were mated and infants 4–9 days old were used in the experiments. The mice were fed with antibiotic-free feed and given water *ad libidum*. The infants remained with their dams for the duration of the studies.

Cultures and growth conditions

Before use as an inoculum, the strains were grown microaerophilically in Brucella Broth (Bacto Brucella Broth, Difco) containing citrated sterile calf blood 5% at 37°C for 30–40 h. Brucella agar with citrated calf blood 5% was used as a nonselective medium; for counting campylobacters from the animal tissues, the medium was supplemented with vancomycin (Vancocin; Eli Lilly Co, Indianapolis, IND) $10\text{ }\mu\text{g/ml}$, trimethoprim (Sigma Chemical Co, St Louis, MO) $5\text{ }\mu\text{g/ml}$, polymyxin B (Sigma) 2.5 IU/ml and cephalothin (Keflin; Eli Lilly

$15\text{ }\mu\text{g/ml}$. All bacterial dilutions were in peptone water 0.1%.

Inoculation of animals with bacteria

A 1.0-ml tuberculin syringe fitted with a blunted 21-gauge needle was used to inoculate each animal intragastrically (i.g.) with *c.* 10^4 campylobacter cells in 0.1 ml of peptone water. The inoculum was made by diluting the brucella broth culture in peptone water and its size was determined by plating in duplicate 10-fold dilutions on brucella blood agar plates.

Endotoxin and iron treatments

Initially, different time intervals from 1 to 24 h between endotoxin injection and campylobacter inoculation were tested. In most cases, endotoxin (from *Escherichia coli* O26: B6) was injected intraperitoneally (i.p.), 6 h before the bacteria (the standard time interval). The endotoxin dose was 4 or $8\text{ }\mu\text{g/animal}$. Iron dextran (Pigfer, Orion Pharmaceuticals, Espoo, Finland), 0.5 mg of iron/animal, was injected i.p. simultaneously with the bacteria. Ammonium ferric citrate, 0.05 or 0.5 mg of iron/animal, was used as an oral iron treatment. The bacterial inoculum was diluted with an aqueous ammonium ferric citrate solution, and the suspension was immediately administered i.g. to the mice. Similarly, iron-free human transferrin (Sigma), 0.1 mg/animal, was administered i.g. to the mice.

Counting of organisms in the tissues

The animals were killed with CO_2 , their abdomens were opened, and their gastrointestinal tracts were removed. Either the whole intestine (from duodenum to rectum) or its separated parts—the small intestine (duodenum, jejunum and ileum) and the large intestine (caecum, colon and rectum)—were homogenised separately in 9 ml of peptone water with a Colworth Stomacher Lab Blender (Tekmar; Cincinnati, OH). In some studies the small intestine was rinsed with 9 ml of peptone water from a 10-ml syringe fitted with a needle similar to that used for the oral inoculation in order to count separately campylobacters associated with the intestinal wall and those in the lumen. The rinsed small intestine was homogenised in the same manner as the unrinsed small intestine. The homogenates were serially diluted in peptone water and plated in duplicate on antibiotic-supplemented brucella blood agar plates. In some experiments, campylobacters were also counted from homogenates of the liver, heart and spleen. After incubation for 48 h at 37°C the number of campylobacter colonies was counted.

Statistical analysis

The campylobacter counts of two or three animals were analysed each time. The statistical significance was assessed with Student's *t* test or the paired *t* test.

Results

Effect of endotoxin on growth of campylobacters

The increases in campylobacter cfu's in the intestines of infant mice over a few days for the three strains 2605, 12650 and KH are presented in figs. 1, 2 and 3. The animals remained colonised with campylobacters for at least 2 weeks. As seen in figs. 1A and 1B, pretreatment of mice with endotoxin had a bacteriostatic effect on campylobacters which lasted for 26–32 h after endotoxin pretreatment. In most experiments, a time interval of 6 h between endotoxin injection and bacterial challenge was used. Endotoxin doses of 4 and 8 μg /animal gave similar bacteriostatic responses. Although the bacteriostatic effect was evident from the cfu determinations for the whole intestine, a more pronounced bacteriostatic effect was seen in the small intestine when the cfu's of small intestine and large intestine were analysed separately (fig. 2). The mean duplication times of campylobacters between 2 and 20 h after inoculation counted in 12 experiments from the results of whole intestines for the three strains, were 4.45 SD 1.5 h and 2.7 SD 0.57 h in endotoxin pretreated and control groups, respectively. These differences were significant ($p < 0.005$). The mean duplication times in five experiments counted similarly for strains 12650 and KH from the cfu's of the small intestine, were 5.0 SD 0.87 h and 2.1 SD 0.24 h in the endotoxin pretreated and control groups, respectively. The differences between these duplication times were again significant ($p < 0.005$). In most cases, as seen in figs. 2 and 3, greater numbers of campylobacter were found in the large intestine than in the small intestine. The preference for large intestine colonisation was even more pronounced when the counts were related to weight or area, as the small intestine weighs more and is much longer than the large intestine.

Time interval between endotoxin pretreatment and campylobacter challenge

Different time intervals from 1 to 16 h between endotoxin pretreatment and bacterial challenge were used to determine the duration of the bacteriostatic effect of the endotoxin. The results of these experiments (see table I) showed that an interval of 1 h was too short and one of 16 h too long for the bacteriostatic effect of endotoxin to be expressed at any time from 2 to 26 h after challenge. Endotoxin injected 3, 4 or 10 h before the bacterial challenge had a significant ($p < 0.01$, $p < 0.02$, $p < 0.05$, respectively) bacteriostatic effect which was

Fig. 1. Growth of *C. jejuni* strains 2605 (A) and 12650 (B) in the intestine (from duodenum to rectum) of infant mice with and without endotoxin (*E. coli* O26: B6; 4 μg /animal i.p.) pretreatment: control groups 1 and 2 $\circ$ — $\circ$; endotoxin group 1 $\blacktriangle$ — $\blacktriangle$; endotoxin group 2 $\bullet$ — $\bullet$; endotoxin + iron dextran (0.5 mg/animal i.p.) $\triangle$ — $\triangle$. Each point represents the mean (SD) of two or three animals. The infecting dose is indicated by the arrow on the vertical axis and the time of endotoxin treatment (E) and the bacterial (C) or iron dextran (Fe) challenge time are indicated by arrows on the horizontal axis.

evident at most of the later assay times but not always in the early period (2–5 h) after challenge.

Reversal of bacteriostatic effect of endotoxin

Oral iron overload with ammonium ferric citrate, parenteral iron overload with iron dextran, and the

Fig. 2. Growth of *C. jejuni* strains 12650 (A) and KH (B) in the small intestine — and the large intestine ---- of infant mice with and without pretreatment with endotoxin (*E. coli* O26: B6; 4 µg/animal i.p.): control group ○; endotoxin group 1 ▲; endotoxin group 2 ●. The infecting dose is indicated by the arrow on the vertical axis and the times of endotoxin treatment (E) and bacterial challenge (C) are indicated by arrows on the horizontal axis.

effect of oral transferrin were tested for ability to reverse the bacteriostatic effect of endotoxin. As shown in table II, reversal of the bacteriostatic effect of endotoxin depended upon the dose of oral iron. A dose of 0.05 mg of iron as ammonium ferric citrate did not reverse the bacteriostatic effect, but one of 0.5 mg of iron as ammonium ferric citrate did.

The effect of parenteral iron dextran on the bacteriostatic effect of the endotoxin is presented in figs. 1A and 3. Viable counts on whole-intestinal

specimens indicated that parenteral iron overload partly reversed the bacteriostatic effect of endotoxin and that the reversal could be delayed and was more evident during the latter part of the bacteriostatic period (fig. 1A). These differences were less clear when counts of duodenum and colon campylobacters were analysed separately (fig. 3). The mean duplication time of campylobacter strains 2605, 12650 and KH in four experiments in the intestine of the mice which were treated with both endotoxin and iron dextran was 4.07 SD 1.7 h. Transferrin caused a similar bacteriostatic effect both in the experimental group dosed with transferrin and in the group pretreated with endotoxin and dosed with transferrin (table II).

Campylobacter counts in the lumen and in the wall of the small intestine

When the campylobacter counts of the contents of the lumen of the small intestine were determined separately from those of the wall of the small intestine, it was found that the counts of the intestinal lumen always correlated with those of the wall and that the intestinal contents contained fewer campylobacters than the intestinal wall. Oral or parenteral iron did not significantly affect the campylobacter counts in the intestinal lumen.

Discussion

Oral inoculation of campylobacters in infant mice caused a transient colonisation which usually lasted for 2 weeks. Because the purpose of the study was to analyse the kinetics of bacterial growth in the intestine at the initial stage of infection, the infectious dose used was low, about 10^4 cells/animal. In the control mice, campylobacter cfu's increased rapidly, the most pronounced increase being seen during the first day after inoculation. Endotoxin pretreatment had a bacteriostatic effect on the intestinal campylobacter counts which was detected during the first day after endotoxin pretreatment. This immediate effect of the endotoxin may be attributed to one or more of the many host effector molecules elicited by endotoxin which result in fever, hyposideraemia, complement activation, interferon production, and the induction of the production of soluble mediators from macrophages and mononuclear cells (Morrison and Ryan, 1987). These events take place a few hours after endotoxin treatment. Since specific antibody production usually starts only after several days, the bacteriostatic effect of the endotoxin in the present study must be associated with acute non-specific

Fig. 3. Growth of *C. jejuni* strain 12650 (A) and KH (B) in the small intestine — or large intestine - - - of infant mice following: ○ no treatment; ▲, ● pretreatment with endotoxin (*E. coli* O26: B6; 8 µg/animal i.p.); △ endotoxin + iron dextran (0.5 mg/animal i.p.). In A the cfu's in the endotoxin-pretreated group were counted between 12 and 30 h, only. The infecting dose is indicated by the arrow on the vertical axis and the time of endotoxin treatment (E), bacterial challenge (C) and iron dextran (Fe) doses by arrows on the horizontal axis.

resistance factors. Of the non-immunological bacteriostatic effects of endotoxin especially well known are fever and hyposideraemia, which are induced a few hours after endotoxin injection and last for about 24 h (Kampschmidt and Upchurch, 1962). The lowered iron saturation level in serum transferrin is associated with a decreased ability of the invading micro-organism to multiply in iron-restricted conditions and thus with a lowered

pathogenicity of the organism for the host. This effect can be eliminated by simultaneous iron injection and saturation of transferrin iron binding capacity (Elin and Wolff, 1974). Endotoxin has been shown to increase the LD50 values by several log units in systemic infections with, for example, salmonellae, neisseriae, *Candida albicans* and mycobacteria (Weinberg, 1984). In mice, LPS pretreatment was shown to prevent the growth of *E. coli*

Table I. Effect of time interval between endotoxin (*E. coli* O26: B6) pretreatment and bacterial challenge on growth of *C. jejuni* strain 12650 in intestine of infant mice

Time (h) between endotoxin pretreatment and bacterial challenge	Challenge dose (cfu)	Treatment of animals	Campylobacters, log ₁₀ cfu, mean (SD) intestine* at different times (h) after bacterial challenge							
			2	5	8	10	12	22	24	26
1	1.1 × 10 ⁴	Control	4.10 (0.10)	...	4.77 (0.27)	...	5.34 (0.33)	...	5.51 (0.30)	
		Endotoxin (4 µg)	4.09 (0.11)	...	4.56 (0.02)	...	5.00 (0.65)	...	5.46 (0.09)	
3	4.5 × 10 ⁴	Control	4.10 (0.16)	4.30 (0.13)	...	5.15 (0.16)	...	6.21 (0.28)	...	6.66 (0.23)
		Endotoxin (4 µg)	3.50 (0.39)	4.04 (0.05)	...	4.30 (0.05)	...	5.20 (0.20)	...	5.70 (0.12)
4	5.7 × 10 ⁴	Control	4.06 (0.15)	...	5.15 (0.16)	...	...	6.21 (0.29)	...	6.71 (0.28)
		Endotoxin (4 µg)	4.30 (0.05)	...	4.29 (0.19)	...	...	5.23 (0.21)	...	5.81 (0.02)
10	3.2 × 10 ⁴	Control	3.75 (0.17)	4.47 (0.10)	5.95 (0.39)	...	6.16 (0.46)	...	6.24 (0.22)	...
		Endotoxin (4 µg)	3.67 (0.19)	4.29 (0.19)	4.80 (0.27)	...	4.95 (0.90)	...	5.65 (0.15)	...
16	1.5 × 10 ⁴	Control	...	4.30 (0.20)	4.86 (0.31)	...	5.35 (0.16)	...	...	...
		Endotoxin (4 µg)	...	4.40 (0.15)	4.98 (0.17)	...	5.5 (0.38)	...	...	...

*Intestine from duodenum to rectum.

Table II. Effect of oral ammonium ferric citrate and transferrin on growth of *C. jejuni* strain 12650 in intestine of endotoxin-pretreated infant mice

Treatment of animals	Campylobacters, log ₁₀ cfu mean (SD)/intestine at different times (h) after bacterial challenge			
	2	6	20	26
Control	3.65 (0.02)	5.96 (0.48)	5.82 (0.20)	5.83 (0.20)
Endotoxin*	2.90 (0.02)	4.44 (0.18)	4.36 (0.20)	4.87 (0.13)
Endotoxin* + ammonium ferric citrate†; 0.05 mg of iron	3.61 (0.4)	3.76 (0.22)	4.04 (0.48)	5.08 (0.15)
Endotoxin* + ammonium ferric citrate†; 0.5 mg of iron	4.20 (0.90)	5.39 (0.01)	5.22 (0.14)	6.37 (0.23)
Transferrin†, 0.1 mg	2.80 (0.05)	3.74 (0.21)	3.53 (0.25)	...
Endotoxin* + transferrin† 0.1 mg	2.25 (0.01)	4.27 (0.15)	3.15 (0.15)	4.10 (0.4)

*8 µg/animal; 6 h before campylobacter challenge.

†Given with campylobacters.

O18: K1 in the peritoneal cavity and blood and to result in a 100-fold increase in LD₅₀ (Vuopio-Varkila *et al.*, 1988).

There are no earlier reports on the effects of systemically injected endotoxin on local infectious agents, such as those on the mucosal surfaces of the mammary, pulmonary, urinary or intestinal epithelium. In the present study, the bacteriostatic effect of endotoxin on intestinal mucosal campylobacters was seen simultaneously with the expected decrease in serum iron (Kampschmidt and Upchurch, 1962). Oral iron overload reversed the bacteriostatic effect of endotoxin. For the reversal, however, a high dose of iron, about 50 mg/kg live weight, was needed. A lower dose of about 5 mg/kg live weight was ineffective. Parenteral iron caused a delayed reversal of the bacteriostatic effect which was seen only about 20 h or more after injection and which was never a total reversal. One reason for this delayed reversal effect may be that systemically administered iron has a more indirect effect on the iron balance at mucosal surfaces than iron administered orally.

As mucus colonisers in mice (Field *et al.*, 1981; Lee *et al.*, 1986), campylobacters have to adapt to

the mucus environment which contains both lactoferrin and transferrin as iron binding compounds (Mason and Taylor, 1978). In studies of experimental and natural mastitis (Harmon *et al.*, 1976), intramammary injections of endotoxin or *E. coli*, caused a 30-fold increase in the amount of lactoferrin produced in the mammary glands. Whether it is the production of iron-binding proteins that is increased or the iron-binding capacity of lactoferrin and transferrin on mucosal surfaces that is changed, is, however, not known. The question of the importance of these phenomena in the bacteriostatic effect of endotoxin needs further research. Transferrin given orally caused a bacteriostatic effect in both control and endotoxin treated animals. The growth of the three campylobacter strains in colostrum bovine milk was also completely inhibited but this inhibition was totally reversed by the addition of ferric ammonium citrate 10 mg/ml (unpublished results). Campylobacters are known to produce both siderophores and outer-membrane proteins (OMPs) probably involved in siderophore binding, when grown *in vitro* under iron restricted conditions, and they can use siderophores produced by other organisms (Field *et al.*, 1986). Further studies are needed to determine whether OMPs and siderophores are produced *in vivo* in mice, especially after endotoxin treatment. On the other hand, iron overloading has been shown to increase the virulence of campylobacters (Kazmi *et al.*, 1984; Stanfield *et al.*, 1987), which also provides evidence of the importance of iron in campylobacter infection.

When campylobacter counts were performed

separately on the small intestine and large intestine, a much stronger colonisation of large intestine was seen. This is in accordance with the results of Blaser *et al.* (1983) and Yrios and Balish (1986) who found the caecum and colon of adult mice to be the most heavily colonised with campylobacters. Although endotoxin treatment caused a significant increase in the duplication time of campylobacters in both the small intestine and in the caecum and colon, the effect was more pronounced in the small intestine. The reason for this is unknown. It may be related to the different effects of endotoxin in different parts of intestinal mucosa, or to the microbial ecology of campylobacters in the intestinal tract, the organism possibly being adapted to caecal and colon mucosal surfaces. Endotoxin is known to decrease iron absorption mediated by transferrin and lactoferrin from the small intestine during the first day after endotoxin treatment (Cortell and Conrad, 1967).

Although in the present study the bacteriostatic mechanism of parenterally added endotoxin could not be fully explained, the effect must be non-immunological, and further research is needed to explain the role of mucosal lactoferrin and transferrin. The present model in which infant mice are pretreated with endotoxin offers a way to analyse non-immunological factors important in the prevention or promotion of colonisation of enteric pathogens.

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