EXPERIMENTAL INFECTION OF GNOTOBIOTIC MICE WITH *CAMPYLOBACTER JEJUNI*: COLONISATION OF INTESTINE AND SPREAD TO LYMPHOID AND RETICULO-ENDOTHELIAL ORGANS

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SUMMARY. Axenic and monoxenic C3H mice were used to develop an animal model for enteroinvasiveness and translocation of *Campylobacter jejuni*. After oral administration of 10^7 – 10^8 viable cells of *C. jejuni* on day 0 (D0), bacterial colonisation was followed quantitatively during 23 days by counting free luminal bacteria and tissue-associated bacteria in the duodenum, ileum and colon. The kinetics of bacterial colonisation were the same in axenic and monoxenic mice; bacteria were more numerous in distal than in proximal intestinal segments.

Electronmicroscope studies of axenic infected mice showed *C. jejuni* free in the intestinal lumen on D2 and D7, and adhering to microvilli or included in enterocyte vacuoles in the colon on D2 without inflammatory reaction; *C. jejuni* was isolated from mesenteric lymph nodes until D23, but from blood, spleen, liver and bile until D1 only. In monoxenic infected mice, *C. jejuni* was found from D1 to D4 in mesenteric lymph nodes and Peyer's patches, whereas the associated bacterium (*Clostridium perenne*) was never cultured from any organs.

On the basis of our observations in this gnotobiotic model, *C. jejuni* appears to be an enteroinvasive bacterium with a particular affinity for lymphoid organs.

INTRODUCTION

Campylobacter jejuni (Véron and Chatelain, 1973) has been recognised as a common bacterial cause of diarrhoea for a few years (King, 1957 and 1962; Butzler *et al.*, 1973 and 1977; Butzler and Skirrow, 1979; Blaser and Barth-Reller, 1981). Epidemiological and clinical aspects of *C. jejuni* enteritis have been well documented (Butzler and Skirrow, 1979; Blaser and Barth-Reller, 1981; Véron, 1981; Skirrow, 1982; Butzler, 1984). Man is usually infected from drinking water or by contact with animal carriers (poultry, dogs, cats, pigs, sheep). Usually there is watery or dysenteric diarrhoea lasting for a few days, but healthy carriers with diarrhoeal relapse are common (Butzler and Skirrow, 1979; Butzler, 1984). A few bacteraemic episodes have been reported (Guerrant *et al.*, 1978; Butzler and Skirrow, 1979; Hanslo *et al.*, 1983;

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Butzler, 1984) as well as appendicitis or mesenteric adenitis with abdominal pain, particularly in children (Pearson *et al.*, 1983; Butzler, 1984).

The pathogenesis of *C. jejuni* diarrhoea is not yet entirely understood. Several animal models have been described but none exactly reproduces the human disease (Newell, 1984); the best are those using newborn animals (Madge, 1980; Prescott *et al.*, 1980; Field *et al.*, 1981; Ruiz-Palacios *et al.*, 1981; Prescott *et al.*, 1982; Soerjadi *et al.*, 1982) or adult rabbits (Removal Intestinal Tie Adult Rabbit Diarrhoea—(RITARD); Caldwell *et al.*, 1983).

Because of clinical and pathological features, the virulence of *C. jejuni* was at first attributed to an enteroinvasive capacity (Butzler and Skirrow, 1979; Price *et al.*, 1979; Butzler, 1984). However, this property was difficult to observe experimentally. The Sereny test (for the strain's ability to produce keratoconjunctivitis in the guinea pig) was always negative (Manninen *et al.*, 1982; Newell, 1984). However, in experiments with either newborn animals (Ruiz-Palacios *et al.*, 1981) or HeLa cells in culture (Manninen *et al.*, 1982; Newell and Pearson, 1982) invasiveness was demonstrated by histological or cytological findings. Moreover, Blaser *et al.* (1983) found an early and sporadic bacteraemia in mice infected orally with *C. jejuni*. However, the route of penetration and the fate of bacteria that have passed through the mucosa are still unclear.

Recently, toxigenic strains of *C. jejuni* were also described (Fernandez *et al.*, 1983; Ruiz-Palacios *et al.*, 1983) so that it is still uncertain what virulence factors are responsible for *C. jejuni* enteritis (Newell, 1984).

To understand better the process of enteroinvasiveness by *C. jejuni*, we chose an in-vivo model consisting of gnotobiotic mice infected orally with *C. jejuni*. Despite their immunological characteristics (Coates *et al.*, 1968), gnotobiotic animals are better models than holoxenic animals because: (a) quantitative estimations performed on unselective media are more precise and easier; (b) the test bacteria can be identified in smears and histological sections; (c) the individual variability due to the large diversity of associated flora in holoxenic animals is virtually absent; and (d) the possible role of associated flora on intestinal colonisation by the test bacteria can be assessed. In gnotobiotic mice, we investigated intestinal colonisation, mucosal invasiveness, and translocation from intestine to Peyer's patches, mesenteric lymph nodes, blood, reticulo-endothelial system and bile.

MATERIALS AND METHODS

Mice. Axenic (germ free) and monoxenic inbred C3H female mice (Centre de Sélection des Animaux de Laboratoire, Orléans, France) were housed in plastic isolators (La Calhène, Paris, France). They were 6–8 weeks old at the beginning of experiments. Food and bedding were sterilised with gamma irradiation; water, cages, and materials for sampling and inoculation were sterilised by autoclaving. Holoxenic mice (with a normal intestinal flora), also of inbred C3H strain, were housed in conventional cages equipped with isocaps.

Eight days before the experiments, monoxenic mice were obtained from a set of axenic mice by giving them, during 24 h, drinking water containing a wild strain of *Clostridium perenne* (c. 10^6 cfu/ml of water).

Before the experiments, faeces were checked for sterility (axenic mice) or intestinal colonisation by *Cl. perenne* only (monoxenic mice). Holoxenic mice were checked to be free of *C. jejuni* and specific enteropathogens.

Bacterial strains and bacteriological methods. *C. jejuni*, strain 79.K, was isolated from the stools of a 2-year-old child with enteritis; this microaerophilic organism grew at 37°C and 42°C,

and was catalase positive. *Cl. perenne*, strain 83.AQ, was isolated from the faeces of a healthy holoxenic mouse; this anaerobic organism was non-motile and produced terminal spores; it did not hydrolyse gelatin nor produce indole; it produced acid from glucose, lactose and maltose, but not from mannitol. Both strains were stored at -80°C in Brucella Broth (BB) (Difco) supplemented by dimethyl-sulphoxide 10%.

These strains were cultivated in BB or on Columbia Agar (CA) (BioMérieux) incubated at 37°C for 48 h in appropriate atmospheric conditions: microaerophilic (O_2 5%, CO_2 10%, N_2 85%) or anaerobic (CO_2 10%, H_2 10%, N_2 80%). Viable bacteria were enumerated by plating 10-fold serial dilutions on CA and incubating for 48 h at 37°C . We verified that the viability of *Cl. perenne* was not significantly reduced by exposure of an inoculum for 1 h to the aerobic conditions of the surrounding atmosphere.

Specimens from animals were cultivated in BB or on CA, except for stools from holoxenic mice which were cultured on Butzler's Campylobacter selective medium (Oxoid).

Oral administration of test strains and observations of mice. Inocula were prepared with isolates of *C. jejuni* obtained 48 h previously from mesenteric lymph nodes removed from an axenic mouse colonised with *C. jejuni*. After overnight fasting, mice were given 1 ml of BB containing 10^7 – 10^8 viable bacteria through a gastric feeding tube. This dose was checked retrospectively.

Blood cells were detected in faeces by phase-contrast microscopy. Diarrhoea was assessed by counting the number of stools recovered on a filter sheet in a cage that a mouse had occupied alone for 1 h.

Intestinal colonisation in axenic and monoxenic mice. Bacterial colonisation was assessed against time, in lumen and washed intestine of three sites: duodenum (the first 2 cm of small bowel), ileum (the last 2 cm of small bowel), and the whole colon. Each intestinal segment was flushed with 5 ml of sterile BB, injected into the lumen with a syringe; free luminal bacteria in this fluid were enumerated by viable count on CA. Further, the gut segments were dissected, extensively washed in eight successive baths of sterile BB, and weighed. Tissue-associated bacteria were enumerated by viable count on CA for each washed segment, after grinding in 5 ml of BB.

Histological and cytological techniques with axenic mice. For electronmicroscopy, fragments of duodenum, ileum and colon were removed, and fixed for 24 h at 4°C in callidine buffer containing glutaraldehyde 2.5%. Samples were then washed in buffer, fixed for 1 h in osmium tetroxide 1%, dehydrated, embedded in Epon (Merck), and cut with an ultramicrotome. Semi-thin sections were stained with toluidin blue; ultrathin sections were contrasted with uranyl acetate and lead citrate, before examination with a Philips EM300 electronmicroscope.

For light microscopy, paraffin-embedded sections were stained with haemalum-eosin.

Bacterial spread in axenic, monoxenic or holoxenic mice. Bacterial spread (translocation) was evaluated by looking for bacteria in blood, bile, spleen, liver, mesenteric lymph nodes and Peyer's patches, sampled in this order to avoid faecal contamination. First, 0.05 ml of blood was collected at the cut tip of the tail. Then mice were killed for laparotomy, and bile was collected with a slender Pasteur pipette. Blood and bile were cultivated in BB in microaerophilic and anaerobic conditions. The spleen and liver were separately ground in 5 ml of BB. Last, the bulk of ileocaecal lymph nodes and one or two Peyer's patches were cut off, washed in two successive baths of sterile BB, and ground in 1 ml of this medium. All homogenates were cultivated in BB and enumerated on CA in microaerophilic and anaerobic conditions.

RESULTS

Clinical and laboratory observations on axenic and monoxenic mice

Axenic and monoxenic mice, 25 of each, were infected orally with 10^7 – 10^8 *C. jejuni* at day zero (D0). The evolution of infection was studied at various intervals (fig. 1), in groups of three mice.

(1) No spontaneous mortality occurred during the 40-day period of observation.

FIG. 1.—Tissue-associated bacteria in intestine of axenic and monoxenic mice infected with *C. jejuni*. At day zero (D0) 25 axenic and 25 monoxenic mice were given 10^7 – 10^8 viable cells of *C. jejuni* orally. Periodically three mice were removed from the isolator for bacterial enumeration (first determination performed 4 h after inoculation). The last four mice were observed until D40. Axenic mice —○—; monoxenic mice —●— (each point is the mean for three mice; SDs ranged from 0.2 to 1.0 $\log_{10}$ cfu/g; the average coefficient of variation was 21%).

(2) The number and nature of stools were followed from D1 to D7 in infected mice and in uninfected control groups (five axenic and five monoxenic uninfected mice housed in separate isolators). In monoxenic groups (D1–D7) and in axenic groups (D2–D7), no significant difference was observed between infected and control groups (4–9 stools/h). However, a mild diarrhoea was observed in axenic groups on D1 only; the stools were unformed and a little more frequent (14.0 ± 6.6 /h in a sample of three mice) than in the control axenic group (6.4 ± 2.0 /h in a sample of five mice).

(3) *C. jejuni* was recovered from the faeces of all axenic and monoxenic infected mice which were examined from D1 to D40.

(4) In axenic infected mice examined from D1 to D40, blood cells were found in faeces from D1 to D4 only (nine mice were observed during this period): red blood cells were found in four mice and leukocytes in one of these four. No blood cells were found either in the monoxenic infected group or in the control groups.

(5) In duodenum, ileum, colon, mesenteric lymph nodes and reticulo-endothelial system, no significant damage or inflammatory reaction were observed by histological examinations performed on D2 and D7 on three axenic and three monoxenic infected mice.

Kinetics of bacterial colonisation of the intestine in axenic and monoxenic mice

Colonisation of the intestine was assessed 4 h after inoculation and at intervals for 23 days by counting the numbers of viable bacteria/g of gut, both free luminal bacteria

TABLE
Detection of *C. jejuni* in specimens from mice after peroral administration of 10^7 – 10^8 viable bacteria

Mice	Time after administration	Proportion of mice (number colonised/number examined) that yielded positive cultures from						
		Peyer's patches	mesenteric lymph nodes	blood	spleen	liver	bile	stools
Axenic	1 h	ND	1/3	1/3	0/3	1/3	2/3	0/3
	1 day	ND	4/6	1/6	1/6	2/6	3/6	6/6
	4 days	ND	5/6	0/6	0/6	0/6	0/6	6/6
	23 days	ND	4/6	0/6	0/6	0/6	0/6	6/6
Monoxenic	1 h	ND	0/2	0/2	0/2	0/2	ND	0/2
	1 day	ND	3/6	0/6	0/6	0/6	0/6	6/6
	4 days	3/7	7/7	0/7	0/7	0/7	0/7	7/7
	23 days	0/4	0/4	0/4	0/4	0/4	0/4	4/4
Holoxenic	1 h	0/3	0/3	0/3	0/3	ND	ND	1/3
	1 day	0/6	0/6	0/6	0/6	0/6	0/6	3/6
	4 days	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	23 days	0/3	0/3	0/3	0/3	0/3	0/3	0/3

ND = Not determined (see text).

and tissue-associated bacteria. Results are shown for tissue-associated bacteria in fig. 1. Counts of free luminal bacteria were similar, but usually 10^1 – 10^2 higher in duodenum and ileum and 10^2 – 10^3 higher in colon, compared with tissue-associated bacteria. The kinetics of bacterial colonisation were similar in the axenic and monoxenic groups; bacteria were more numerous in distal than in proximal intestinal segments.

In the monoxenic group, the numbers of the associated strain (*Cl. perenne*) remained steady during the experiment, both for free luminal bacteria (duodenum, $< 10^2$; ileum, 10^5 – 10^7 ; colon, 10^8 – 10^9 /g of gut) and for tissue-associated bacteria (duodenum, $< 10^2$; ileum, 10^4 – 10^7 ; colon, 10^5 – 10^6 /g of gut).

Bacterial translocation from the intestine in axenic, monoxenic and holoxenic mice

To study the possibility of bacteraemia resulting from upper gastrointestinal trauma by the inoculation procedure, blood culture was performed in each infected animal 15 min after inoculation. These blood cultures remained negative.

Specimens from various parts of the body (table) were cultured 1 h after oral administration of *C. jejuni* and on D1, D4 and D23. *C. jejuni* was recovered from the stools of axenic and monoxenic infected mice from D1 to D23. It was recovered also from various organs, but the viable counts (/g) were low: 10^2 – 10^4 for spleen and liver, 10^1 – 10^2 for Peyer's patches, and 10^2 – 10^3 for ileo-caecal lymph nodes.

In axenic mice, Peyer's patches were always too small to be excised; but *C. jejuni* was found in the majority of mesenteric lymph nodes sampled from D1 to D23; and it was cultivated irregularly from the other sites (blood, spleen, liver and bile) 1 h after inoculation and on D1, but not later. In monoxenic mice, Peyer's patches became enlarged and colonised at D4, mesenteric lymph nodes were colonised from D1 to D4, but no growth was obtained from the other sites; the associated strain (*Cl. perenne*) was

never grown from any organs or tissues. In holoxenic mice, *C. jejuni* was recovered from the stools until D1 but no later; it was never grown from other sites, nor was intestinal flora grown from blood or organs.

Microscopic evidence of enteroinvasiveness in axenic mice

Electronmicroscopy on D2 and D7 revealed differences in the location of *C. jejuni*. At D2, several bacteria were observed free in the mucous environment of colonic epithelium, some of them near the surface of a goblet cell (fig. 2a) or near enterocyte microvilli (fig. 2b), others in close association with microvilli (fig. 2c) or on an epithelial surface devoid of microvilli (fig. 2d). A few bacteria were included in enterocyte vacuoles; some of them looked partly lysed (fig. 2e), while others retained their size, characteristic S-shape and outer membrane (fig. 2f). At D7, only free luminal bacteria were noted.

DISCUSSION

Our results show that the kinetics of intestinal colonisation by *C. jejuni* are similar in axenic and monoxenic mice: *C. jejuni* colonised the intestine for at least 23 days, particularly the colon and ileum (fig. 1), and was recovered from the faeces for 40 days. Conversely, *C. jejuni* could be recovered from the stools of holoxenic mice for less than 4 days (table). These results suggest that commensal flora provide an effective "barrier" against intestinal colonisation by *C. jejuni*. It is thus likely that *C. jejuni* enteritis occurs preferentially in man when the intestinal flora is disturbed.

Electronmicroscopic examinations demonstrated that some of the tissue-associated *C. jejuni* were embedded in the surrounding mucus (fig. 2b), and adherent to intestinal mucosa (fig. 2c), suggesting that adherence would be the first step of invasiveness. Moreover, many bacteria were intracellular (figs. 2e and f), indicating an invasive property. In our model, *C. jejuni* endocytosis occurred without severe damage to intestinal cells, and some bacteria appeared to be still alive in the cytoplasm (fig. 2f). Invasiveness of *C. jejuni* has been observed with the electronmicroscope by others also, either with cultivated cells (Newell and Pearson, 1982) or in the new-born holoxenic chicken (Ruiz-Palacios *et al.*, 1981). The latter study showed an enterocyte membrane disruption associated with cell penetration by *C. jejuni*. In this case, the role of a toxin, associated with the infecting strain, could be considered. In our model, the absence of severe intestinal damage is consistent with the absence of obvious clinical disturbance.

Spread of *C. jejuni* from the intestine was observed both in axenic and in monoxenic mice but never in holoxenic mice, probably because of the intestinal commensal flora in the latter. The early bacteraemia that was observed in some only of the axenic mice, 1 h and also 1 day after oral inoculation, was not due to gastrointestinal trauma from the inoculation procedure. Bacteria were found also in spleen, liver and bile, and their rapid disappearance could be due to nonspecific bactericidal factors such as complement or phagocytic cells. However, translocation occurred from the intestinal tract to lymphoid organs, until D23 in axenic mice and D4 in monoxenic mice. Possibly, destruction of bacteria in these lymphoid organs may be related to a specific immune response. In our monoxenic model, the associated strain (*Cl. perenne*) was never found in any tissue, whereas *C. jejuni* did translocate to mesenteric lymph nodes,

FIG. 2.—Electronmicrographs of colonic epithelium of axenic mice, 2 days after oral administration of *C. jejuni* (ultra-thin sections stained with uranyl acetate and lead citrate). Bar = 1 μ m. Bacteria (arrowed), with diameters ranging from 0.2 to 0.5 μ m, are seen: (a) near the surface of a goblet cell; (b) near epithelial microvilli; (c) in close association with microvilli; (d) on an epithelial surface devoid of microvilli; (e) and (f) in vacuoles within epithelial cells, some bacteria looking partly lysed, others retaining their size, characteristic S-shape, and outer membrane.

possibly because it possesses a specific property required for translocation (Steffen and Berg, 1983). Early bacteraemia has already been reported in man (Guerrant *et al.*, 1978; Pearson and Skirrow, 1983), in holoxenic mice (Blaser *et al.*, 1983) and in adult rabbits (Caldwell *et al.*, 1983); however, translocation to the reticulo-endothelial system and lymphoid organs has apparently not been reported previously.

Our experimental model of *C. jejuni* enteritis in mice shows a limited spread of this enteroinvasive microorganism in the tissues. The results are consistent with clinical reports of benign infection in patients with normal immunological status. However, the lymphoid tropism of bacteria, observed in this experimental infection, might explain the occasional observations of appendicitis, mesenteric adenitis or painful abdominal syndromes related to *C. jejuni* infection (Butzler and Skirrow, 1979; Pearson and Skirrow, 1983; Pearson *et al.*, 1983). Nevertheless, the invasive mechanism described in this work does not preclude the possible role of an enterotoxin, especially in cases of watery and dysenteric diarrhoea.

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