Invasive and enterotoxic properties in *Campylobacter jejuni* and *Campylobacter coli* strains isolated from humans and animals

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Invasive properties of 15 strains of thermotolerant ***Campylobacter*** *(12* ***C. jejuni*** *and 3* ***C. coli****) were studied using HeLa cells cultures. In four of them (3* ***C. jejuni*** *and 1* ***C. coli****), randomly selected, intestinal perfusion experiments were conducted in order to asses enterotoxigenicity. All strains were able to invade HeLa cells. The number of invaded HeLa cells ranged from 3 to 46%. In addition to their invasive properties, the 4 strains used in perfusion experiments were able to induce either a net secretory flux or an impaired sodium transport.*

Key words: ***Campylobacter****, enterotoxigenicity, invasion, virulence factors.*

INTRODUCTION

Campylobacter jejuni and *C. coli* are zoonotic bacteria world-wide known as important agents of human diarrhea. Enteric infections due to these organisms are highly prevalent among children, specially in developing countries (Skirrow, 1982; Blaser *et al, 1983; Glass* et al, 1983; Fernández, 1992).

Intestinal infection can develop as an exudative process with blood and leukocyte in stools (Blaser *et al,* 1979), or as a watery secretory diarrhea (Glass *et al,* 1983), suggesting that invasion and production of enterotoxic substances are the mechanisms by which *C. jejuni* and *C. coli* cause diarrhea. With this respect, it has been previously demonstrated by Fernández *et al (1983)* and Ruiz-Palacios *et al* (1983) that these bacteria produce enterotoxic substances, which induce a secretory response in rat jejunal segments.

The aim of this study was to evaluate invasiveness and enterotoxic activity of *C. jejuni* and *C. coli* strains isolated from normal children, patients with diarrhea and healthy animals from different species.

MATERIAL AND METHODS

Bacterial strains

Fifteen *Campylobacter* strains were tested for *in vitro* epithelial invasiveness studies; 4 of them were randomly selected for evaluation of enterotoxic activity. The sources from which strains were isolated are shown in Table I.

All strains were initially isolated on Butzler medium (Butzler and Skirrow, 1979) and identified by standard tests (Lior, 1984). Twelve strains corresponded to *C. jejuni* and three to *C. coli.*

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TABLE I

Invasion of *C. jejuni* and *C. coli* in HeLa cells

Species	Source	% of invaded cells	*Campylobacter*/ invaded cell (mean ± SEM)
C. jejuni	D	23	15.0 ± 14.0
C. jejuni	D	33	24.5 ± 13.7
C. jejuni	D	4	34.6 ± 25.5
C. jejuni	D	8	50.2 ± 35.4
C. jejuni	D	10	5.1 ± 2.6
C. jejuni	D	20	9.2 ± 4.5
C. jejuni	CH	4	8.5 ± 4.4
C. jejuni	N	9	6.2 ± 3.6
C. jejuni	N	16	12.5 ± 8.2
C. jejuni	DOG	10	7.3 ± 3.2
C. jejuni	HEN	8	7.1 ± 3.0
C. jejuni	CAT	46	9.1 ± 7.9
C. coli	N	5	14.8 ± 6.6
C. coli	DOG	20	9.2 ± 6.4
C. coli	CAT	3	23.0 ± 12.2

D = Acute diarrhea
N = Normal children
CH = Chronic diarrhea

In vitro invasion studies

In vitro invasiveness studies were carried out in HeLa 219 cells. Cells were cultured overnight on coverslips, in Leighton tubes containing minimum essential medium with 10% fetal calf serum (MEM-10%), at 37°C under 5% CO_2 atmosphere. The medium was replaced with 1 ml *Campylobacter* suspension ($6x10^8$ colony forming units) in MEM with 2% fetal calf serum. Cells were incubated for 3 h at 37°C and 5% CO_2, washed 10 times in PBS and reincubated for 4 h with 1 ml MEM-10%. Following this incubation, coverslips were washed 3 times with PBS, fixed with methanol, stained with May-Grünwald Giemsa, mounted and examined under oil-immersion light microscopy (1000X). The rate (N° of invaded cells/total cells examined x 100) of invasion and the number of bacteria observed in each invaded HeLa cell were determined counting at least 200 cells, and the number of invading bacteria estimated in a minimum of 35 invaded HeLa cells.

Enterotoxin production studies

Enterotoxin production studies were carried out by perfusion of jejunal segments of adult Wistar rats with culture supernatants of 4 *Campylobacter* (3 *C. jejuni* and 1 *C. coli*) strains as previously described (Fernández *et al*, 1983). Supernatants were prepared by individually culturing the *Campylobacter* strains in Brucella broth supplemented with 5% defibrinated sheep blood at 37°C under a reduced atmosphere obtained with the GasPak system without the catalyst. After 72 h of incubation, the cultures were centrifuged under refrigeration at 6,000 rpm for 15 min and filtered through Millipore membranes (pore size 0.22 μm). Next, pH and sodium and glucose concentrations in the supernatants were adjusted to 7.0; 130 mEq/liter and 130 mg/100 ml, remaining the osmolarity at 300 mOsm/liter. Brucella broth and the culture supernatant of the *Escherichia coli* K-12 J53 strain were used as negative controls. The later was prepared under the same conditions used for *Cam-*

pylobacter supernatants. Polyethylene glycol (PEG) was used as a nonabsorbable marker at a concentration of 0.6 g/liter in supernatants and in negative control solutions.

Perfusion experiments were carried out using male Wistar rats, about 150 to 200 g, that were made to fast overnight but were allowed to drink water. Four rats were perfused with each solution, producing 25 to 36 perfusate samples. Each rat was anaesthetized with intraperitoneal injection of pentobarbital (40 mg/kg), the abdominal cavity opened by a mid-line incision and the jejunum was isolated. A polyethylene tube was inserted through a small incision made in the jejunum and fixed in place with a ligature. A jejunum segment of 30 to 40 cm was utilized, and its distal section was also cannulated. The intestinal lumen was flushed out with warm isotonic saline solution, taking care to avoid distending the experimental segment. The proximal cannula was connected to a Harvard 1210 peristaltic pump, and the solutions were perfused during 120 min at a rate of 0.6 to 1.2 ml/min. The first 30 min of the perfusion period was allowed for equilibration conditions to be reached. The second period was used to collect perfusate specimens, one sample each 10 min. At the end of the experiment, the animal was sacrificed, the jejunum removed and the experimental loop measured. Sodium and PEG concentrations were determined in each perfusate sample by standard flame spectrophotometry and by the method described by Skoog (1979), respectively. Net transport was calculated from changes in PEG and individual solute concentrations by the usual water-marker technique equations (Power and Malawer, 1968). A transport value for each sample was calculated and the mean transport rates ± standard errors of the mean were determined for each test group. The significance of differences in means was determined by the Student's *t* test for independent means.

RESULTS

Results of the *in vitro* invasiveness studies are shown in Table I. All the strains were able to invade HeLa cells with rates ranging from 3 to 46% of cells in culture. The number of bacteria per invaded HeLa cell varied from 5.1 ± 2.6 to 50.2 ± 35.4. Figure 1 shows a HeLa cell containing typical forms of *Campylobacter* inside an intracytoplasmic vacuole.

TABLE II

Effect of *C. jejuni* culture supernatants on sodium transport in rat jejunum *in vivo*

Perfused solutions	Isolation source	Sodium transport mean ± SEM[a, b] µEq/min/cm
SUPERNATANTS		
C. jejuni	Human acute diarrhea	−130.4 ± 124.2*
C. jejuni	Human chronic diarrhea	+42.4 ± 48.3*
C. jejuni	Dog feces	−510.5 ± 394.8*
C. coli	Normal human feces	−365.2 ± 277.7*
NEGATIVE CONTROLS		
Brucella broth		+127.1 ± 78.2
E. coli K-12		+174.4 ± 34.5

[a] number of perfusate samples analyzed: 25 to 36.
[b] +, absorption; –, secretion.
* $p < 0.01$, when compared with negative controls.

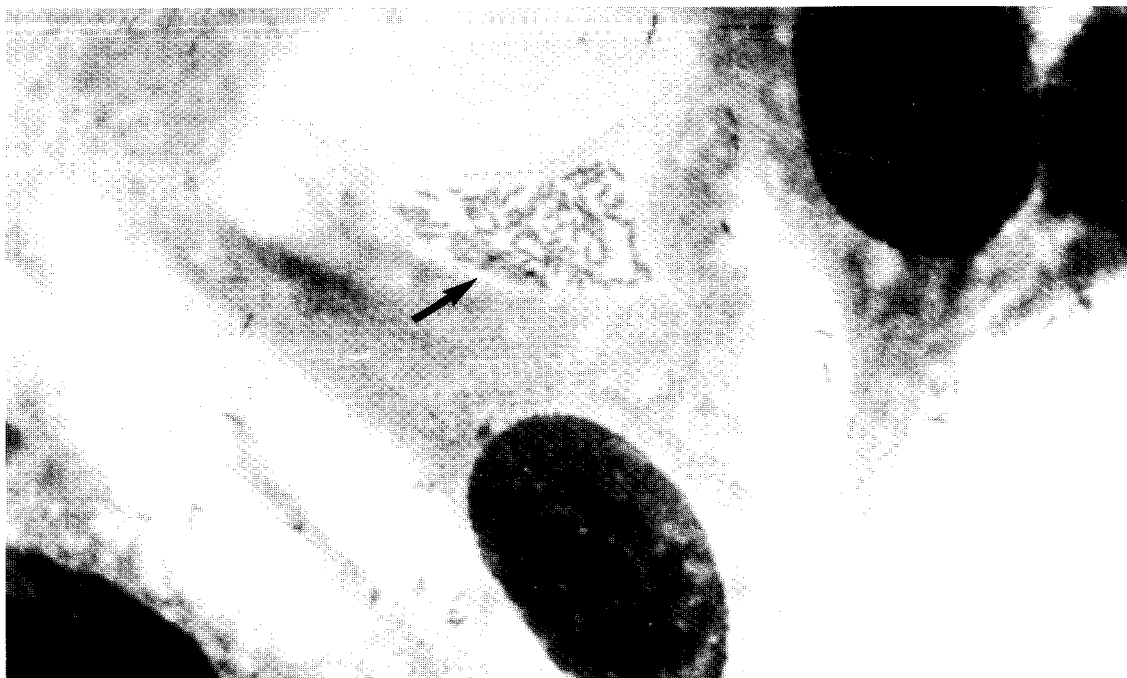

Fig 1. HeLa cell showing internalized bacteria (arrow) inside a vacuole.

Results obtained from the perfusion experiments are shown in Table II. A net sodium secretory flux (plasma-lumen) was observed in animals perfused with the culture supernatants of three strains. One of the four strains tested, corresponding to a *C. jejuni* strain isolated from a case of chronic diarrhea, showed a discrete absorption flux (lumen-plasma). However, when this effect was compared with that of the perfusion solutions used in the control experiments, the former was found to significantly affect sodium transport.

DISCUSSION

The invasion of tissue cultured cells and the ability to interrupt the intestinal transport of electrolytes in experimental biological models, correlates well with the *in vivo* invasiveness and enterotoxin production capacities of enteropathogenic bacteria (Klipstein *et al,* 1976; Neter, 1982).

In this study we attempted to demonstrate invasiveness and enterotoxigenicity of *C. jejuni* and *C. coli* in HeLa cells and rat jejunal perfusion experiments, respectively.

All the strains tested, regardless their isolation source, were able to invade HeLa cells. These results are in agreement with those reported by Manninem *et al (1982), Newel and Pearson (1984) and Fauchere* et al (1992), except for the fact that the number of bacteria observed inside the cells was higher in this study. A possible cause for such findings could be the more extended second incubation period (4 h) used in our experiments. This period, usually of 3 h length, allows bacterial invasion of the cells (Konkel and Joens, 1989).

The supernatants of three out of four cultures induced net sodium secretory fluxes. The supernatant of a *C. jejuni* strain, isolated from a chronic diarrheal process, induced an impaired sodium transport. Previous studies using perfusion techniques demonstrated that enterotoxic substances produced by *Staphylococcus aureus* (Sullivan and Asano, 1971), *Clostridium perfringens* (McDonel, 1974) and *Escherichia coli* (Klipstein *et al,* 1976) were able to induce a net secretory process of water and electrolytes.

The results obtained in this work confirmed our earlier findings (Fernández *et al,* 1983) and are in agreement with the

accumulation of fluids observed by Ruiz-Palacios *et al* (1983) in rat intestinal loops challenged with enterotoxic *Campylobacter* strains, previously verified by CHO cells tests. Enterotoxigenicity in *Campylobacter* strains was also confirmed by Johnson and Lior (1986) and by Klipstein *et al* (1986) in tissue cultures and in rat intestinal loop test.

The 4 strains studied were able to invade HeLa cells and to induce a sodium secretory flux, providing evidence of the co-existence of both toxigenic and invasive properties in *Campylobacter* strains. These findings are in agreement with those of Butzler and Skirrow (1979), Ruiz-Palacios *et al* (1983), Newel and Pearson (1984) and Klipstein *et al* (1986).

All *Campylobacter* strains isolated from animals showed to be either invaders or invader-toxin producers, suggesting that these pathogenic mechanisms could be present in strains coming from their natural reservoirs. This observation reinforces the importance of the animal hosts as contaminating sources for humans (Blaser *et al,* 1983; Fernández, 1992).

The existence of these virulence factors in strains isolated from healthy children suggests that host-related factors could play an important role in the development of a clinical response to infection with pathogenic strains of *Campylobacter*, as stated by Blaser *et al* (1985).

Klipstein *et al* (1985) were unable to demonstrate any pathogenic properties in *C. jejuni* strains isolated from asymptomatic persons. In India, however, enterotoxigenic strains were isolated with high frequencies from symptomatic and asymptomatic children (Mathan *et al,* 1984).

These observations as well as the coexistence of both mechanisms of pathogenicity in *Campylobacter* strains isolated from humans with and without diarrhea, and from animals and environmental sources, need further studies in order to assess the pathophysiology of enteric infection due to *Campylobacter* species.

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