Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines

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SUMMARY

The biochemical and biological properties of the flagella of *Campylobacter jejuni* have been investigated using two variants selected from a flagellate, motile clinical isolate (strain 81116): a flagellate, non-motile variant (SF-1) and an aflagellate variant (SF-2). Phenotypic and biochemical analysis of the strains and amino acid analysis of the isolated flagella suggest that the variants differed from the wild-type strain only in the absence of flagella and/or motility. The aflagellate variant poorly colonized the gastrointestinal tract of infant mice but the flagellate, non-motile variant colonized the mice as successfully as the wild-type strain. ³⁵S-labelled organisms were used to investigate the attachment of the variants to human epithelial cell monolayers *in vitro*. The flagellate, non-motile strain attached more efficiently to the cells than the wild-type strain or the aflagellate strain. Differences in attachment suggest that an adhesin is intimately associated with flagella of *C. jejuni* and that active flagella mediate only a tenuous association with host cells. This adhesin attached most efficiently to cells of intestinal epithelial origin and was not specifically inhibited by various sugars.

INTRODUCTION

Campylobacter jejuni is a major cause of human acute bacterial gastroenteritis (Skirrow, 1982). Although the mechanisms by which *C. jejuni* infections induce diarrhoea are still under investigation recent studies indicate that attachment and invasion (Manninen, Prescott & Dohoo, 1982; Newell & Pearson, 1984) are involved.

Enteropathogenic bacteria must overcome a number of efficient host defence mechanisms in order to establish a successful infection. In particular, the mucus layer quickly eliminates unattached organisms from the gastrointestinal tract. Motile bacteria may traverse the mucus layer and survive in the relatively stagnant regions under the mucus, close to the epithelial cells or in the crypts.

Additionally, contact with the intestinal epithelium may be enhanced by adhesin-mediated attachment. Studies on *Vibrio cholerae* indicate that motility is associated with virulence (Guentzel & Berry, 1975). However, the role of flagella in the pathogenesis of cholera may involve attachment as well as motility (Attridge & Rowley, 1983).

Adhesive structures, such as pili, have not been observed on campylobacters (Dijs & De Graaf, 1982), but *C. jejuni* has bi-polar flagella, up to 4 μm in length, which confer a characteristic darting motility (Butzler & Skirrow, 1979). Such prominent structures may establish initial contacts with host tissue and thereby mediate adhesion. Previous studies have shown that the flagella of *C. jejuni* are intimately associated with the surface of tissue culture epithelial cells during attachment (Newell & Pearson, 1984).

In order to establish the role of flagella in the virulence of *C. jejuni* two non-motile variants (one flagellate and one aflagellate) were selected from a clinical isolate. Both the wild-type strain and the variants colonized the gut of intragastrically inoculated infant mice, but marked differences were observed in the period and extent of colonization. These results suggested that flagella, active or inactive, were essential for successful colonization. The same strains were then studied in *in vitro* assays of attachment in order to investigate the role of flagella in adhesion.

MATERIALS AND METHODS

Bacterial strains

The wild-type strain (*C. jejuni* 81116) was isolated from the stool of a patient with diarrhoea on Skirrow's antibiotic selective medium (Skirrow, 1977) at 43 °C under microaerophilic conditions.

The selection of non-motile variants from *C. jejuni* has previously been described (Newell, McBride & Pearson, 1984). Briefly, non-motile organisms were enriched in the population by sequential subculture from the centre of semi-solid nutrient gelatin agar (1 % peptone, 0.33 % yeast extract, 0.5 % sodium chloride, 0.8 % gelatin and 0.75 % agar) stab cultures. Non-motile colonies were identified by the type of growth on plates of nutrient gelatin agar containing 1 % agar (motility agar) and by dark-ground microscopy.

Colonies of non-motile organisms were blotted on to nitrocellulose paper (Schleicher & Schull, Keene). Endogenous peroxidase was blocked by incubation of the wet blots in methanol containing 0.5 % (v/v) hydrogen peroxide (100 vol). Non-specific binding was eliminated by incubation of the nitrocellulose blots in 3 % bovine serum albumin in 10 mM Tris, 0.9 % sodium chloride, pH 7.4, for 2 h at 37 °C. Colonies of flagellate and aflagellate bacteria were differentiated by incubation for 2 h at 37 °C in 1:100 dilution of rabbit anti-flagella antiserum (Newell, McBride & Pearson, 1984) followed by 1:1000 dilution of peroxidase conjugated goat anti-rabbit IgG antiserum (Miles Research Laboratories, Slough). The antisera were diluted in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM sodium chloride, 5 mM EDTA, 0.25 % gelatin and 0.05 % (v/v) Nonidet P-40. The unbound protein was removed by extensive washing in 50 mM Tris-HCl buffer, pH 7.4, containing 1 M sodium chloride, 50 mM EDTA, 0.25 % gelatin and 0.4 % sodium lauroyl sarcosine. The presence of peroxidase was detected using *O*-dianisidine as the substrate.

The flagellate, non-motile strain used in these studies was designated SF-1. The aflagellate strain used was designated SF-2. These variants were cloned six times to confirm stability. All strains were stored in 10% (v/v) glycerol in 1% proteose peptone in liquid nitrogen and subcultured only once before use.

Characterization of the variants: The strains were biotyped according to the method of Skirrow & Benjamin (1980) and serotyped by the passive haemagglutination technique of Penner & Hennessy (1980) and the slide agglutination technique of Lior *et al.* (1982).

Sarkosyl-insoluble outer membrane and flagella preparations from the wild-type strain and variants were prepared as previously described (Newell, McBride & Pearson, 1984).

Amino acid analysis

The amino acid composition of flagella preparations from strains 81116 and SF-1 were determined using either HCl hydrolysis or 2-mercaptoethane sulfonic acid hydrolysis. Samples (50 µg) of flagella were hydrolysed in evacuated tubes at 110 °C for 18 h with 6 M-HCl or for 24 h with 3 N 2-mercaptoethane sulphonic acid (Penke, Ferenczi & Kovacs, 1974). After hydrolysis the HCl was removed by repeated desiccation and rehydration. Samples hydrolysed with 2-mercaptoethane sulphonic acid were neutralized with 10 N NaOH. Amino acid analysis was performed on a Rank-Hilger Chromaspek amino acid analyser. Amino acid recoveries were calibrated by parallel hydrolysis of egg white lyzozyme.

Colonization of infant mice

Three to 5-day-old Balb/c mice were inoculated either intragastrically with organisms suspended in phosphate-buffered saline (Newell & Pearson, 1984) or orally with organisms suspended in boiled cow's milk. The method of inoculation did not appear to affect the colonization. The separation of the infants from their mothers for 4–5 h, including a period before and after inoculation, did not influence the results; later experiments, therefore, were performed on unseparated mice. The inoculum was 1×10^4 to 1×10^7 c.f.u. in 50 µl of the wild-type strain or the variants. Sections of the gut (from mice up to 14 days old) were homogenized as previously described and the viable counts expressed as the numbers of organisms per total gut. Faecal pellets (from mice over 14 days old) were collected, weighed and homogenized. Viable counts were determined on antibiotic selective medium (Skirrow, 1977) containing 2% agar and expressed as the number of organisms per gramme of faeces. Recovered organisms were monitored for motility by colony morphology on motility agar and for flagella by transmission electron microscopy.

Radiolabelling of bacteria

All strains were grown for 12 h at 37 °C in a defined growth medium (DGM) based on Dulbecco's modification of Eagle's medium (Smith *et al.* 1960) modified to give 200 µg ml⁻¹ of each amino acid but excluding methionine. This was added as 10 µCi ml⁻¹ [³⁵S]methionine (Amersham International) to the medium before inoculation. The specific activity of the bacterial suspension (1×10^8 c.f.u. ml⁻¹) was 0.05–0.1 µCi ml⁻¹. Preliminary viability studies confirmed that ³⁵S incor-

poration was directly related to the number of bacteria and that there was no significant loss of bacterial viability or differential incorporation of [^{35}S]methionine between the strains.

Attachment to tissue culture cells

The epithelial cell lines (HeLa 229, HEp2 and INT 407 (Flow Laboratories Ltd., Irvine) were grown as monolayers in Eagle's Minimal Essential Medium (MEM) (Gibco Europe Ltd, Paisley) with 10% fetal calf serum, benzyl penicillin (100 units ml^{-1}) and gentamicin (10 $\mu\text{g ml}^{-1}$). Cells at a concentration of $4-5 \times 10^5$ cells/well were grown in 24-well trays in medium without antibiotics, overnight at 37 °C to give a lightly confluent monolayer. The medium was replaced with 0.5 ml of ^{35}S -labelled bacterial suspension (2×10^8 c.f.u. ml^{-1}) and the trays for 4 h at 37 °C in 5% carbon dioxide. Unattached bacteria were removed by washing three times in Hank's Basal Salt Solution. The monolayers were then solubilized in 1% SDS in 0.1 M sodium hydroxide and the radioactivity associated with the cells was measured by scintillation counting. The percentage attachment was determined as the c.p.m. bound to the monolayer over the c.p.m. added to the monolayer $\times 100$. A one-tailed Student *t* test was used to indicate significant differences.

Inhibition of attachment

The effect of various carbohydrates on the attachment of the [^{35}S]SF-1 strain to INT 407 cells was investigated. The bacteria were suspended in DGM containing galactose, glucose, fucose, mannose, *N*-acetyl glucosamine, *N*-acetyl galactosamine or glucitol (all from Sigma Ltd) at concentrations of 0.1–50 mM. The percentage inhibition of attachment was determined as the c.p.m. bound to the monolayer in the presence of the carbohydrate over the c.p.m. bound to the monolayer in the control $\times 100$.

RESULTS

Characterization of the variants

The phenotypic analysis of the wild-type strain and the variants showed that they were all catalase- and oxidase-positive, all hydrolysed hippurate, produced hydrogen sulphide and were sensitive to nalidixic acid. All strains were serotype 6 by the passive haemagglutination technique for heat-stable antigens. Strains 81116 and SF-1 were serotype 6 and SF-2 untypable by the slide agglutination technique for heat-labile antigens. The colonies of the wild-type were spreading whilst those of the variants were compact. The wild-type organisms had a characteristic darting motility by dark-ground microscopy but the organisms of both variants were non-motile.

A comparison of the outer membrane proteins of the three strains indicated that a 62 kDa major protein and a 84 kDa minor protein were absent from the outer membrane preparation of the aflagellate variant (Fig. 1). Flagella isolated from both the wild-type and SF-1 strains had identical PAGE profiles with a major 62 kDa protein and a minor 84 kDa protein. The amino acid analysis of these two flagella preparations (Table 1) showed only minor differences.

Fig. 1. SDS-PAGE of outer membrane preparations and flagella from *C. jejuni* 81116 and variants: track 1, isolated flagella; track 2, outer membrane preparation from wild type strain (81116); track 3, outer membrane preparation from non-motile, flagellate strain (SF-1); track 4, outer membrane preparation from aflagellate strain (SF-2).

Colonization of infant mice

Strain 81116 colonized the gastrointestinal tract of 4 to 5-day-old mice, with a significant increase in the number of organisms recovered compared to the inoculum, but did not cause deaths or diarrhoea. Colonization of the stomach, duodenum and upper small intestine was brief, lasting 1–3 days. However, campylobacters were recovered from the lower small intestine, caecum and colon for up to 15 days and were detected in faecal pellets up to 31 days post-infection (Fig. 2a). Colonization of the colon with strain 81116 occurred with as few as 3×10^4 c.f.u. per mouse.

The aflagellate strain (SF-2) poorly colonized infant mice with no increase in the number of organisms over the inoculum (Fig. 2b). When present, organisms were restricted to the caecum and small intestine and organisms were not recovered from the gastrointestinal tract or the faecal pellets after 7 days post infection.

Table 1. *The amino acid composition of flagella isolated from strains 81116 and SF-1*

Amino acid	No. residues/molecule		Mole (%)	
	81116*	SF-1†	81116	SF-1
Aspartic acid	90	87	16.5	15.9
Threonine	55	52	8.8	8.3
Serine	71	78	9.8	10.8
Glutamic acid	48	47	9.9	9.6
Glycine	71	81	6.4	7.3
Alanine	68	67	7.7	7.6
Valine	34	33	5.4	5.2
Methionine‡	11	11	2.3	2.3
Isoleucine	48	43	8.6	7.7
Leucine	39	39	7.0	7.0
Tyrosine	9	10	2.3	2.6
Phenylalanine	19	21	4.5	4.9
Lysine	33	32	6.7	6.5
Histidine	0	0	0	0
Arginine	16	17	4.0	4.2
Half-cysteine	0	0	0	0
Tryptophan	0	0	0	0
Proline	0	0	0	0

* Mean of six experiments. † Mean of two experiments. ‡ Determined by hydrolysis with 3 N 2-mercaptoethane sulphonic acid.

Colonization of the infant mice with the non-motile, flagellate strain (SF-1) was generally for a shorter time (up to 14 days) than with the wild type but about 50% of the mice showed a resurgence of infection between 18 and 22 days post infection detectable by organisms in the faecal pellets (Fig. 2c). Colonization occurred in the middle and lower small intestine as well as the caecum and colon and was established with as few as 1×10^4 organisms.

Organisms recovered from tissue and faeces showed the same phenotype as the challenge inoculum. Moreover, darkground microscopy of intestinal lumen contents recovered 3 days after challenge showed that SF-1 and SF-2 were non-motile *in vivo*.

Attachment to tissue culture cells

Organisms of the non-motile, flagellate variant (SF-1), labelled with [^{35}S]methionine, were incubated with monolayers of INT 407 cells for various time intervals in order to determine the appropriate incubation time. There was a steady increase in the percentage attachment with time followed by a rapid decline after 5 h of incubation (Fig. 3). A 4 h incubation period was chosen for subsequent assays.

The attachment of the three strains to INT 407, HEp2 and HeLa cells is shown in Table 2. In each experiment the attachment of bacteria to all cells was relatively low but strain SF-1 attached significantly better than strain 81116 or SF-2 ($P < 0.01$) to INT 407 and HeLa cells. Moreover there was a significant increase in attachment of strain SF-1 to INT 407 cells compared with HeLa or HEp2 cells.

Fig. 2. The colonization of infant mice with *C. jejuni* 81116 and variants: 5-day-old mice were intragastrically inoculated with 1×10^7 c.f.u. (A) Wild-type strain (81116), (B) aflagellate strain (SF-2), (C) non-motile, flagellate strain (SF-1). For 1–12 days post-inoculation viable counts are given for the whole gut as the median value and the range. Six mice were harvested for each point. After 13 days post-inoculation, viable counts are given per gram of faecal pellet for individual mice. Viable counts below 1×10^3 c.f.u. are given as $< \log 3$.

Fig. 3. Time course of attachment of ^{36}S -labelled strain SF-1 to INT 407 cell monolayers.

Table 2. *The percentage attachment of bacterial strains to tissue culture cell monolayers*

Cell line	81116	SF-1	SF-2
INT 407	0.28 ± 0.006	1.3 ± 0.3	0.25 ± 0.02
HEp 2	0.25 ± 0.02	0.21 ± 0.02	0.2 ± 0.03
HeLa	0.25 ± 0.04	0.55 ± 0.04	0.02 ± 0.02

* Standard deviation of the mean calculated from six experiments.

The inhibition of the attachment of SF-1 to INT 407 cells by various carbohydrates was also investigated. Mannose demonstrated a concentration-dependent inhibition of attachment increasing to 53 % inhibition at a concentration of 50 mM. This attachment was also inhibited by the same concentration of galactose (34 %), glucose (40 %), fucose (24 %), *N*-acetyl glucosamine (41 %) and *N*-acetyl galactosamine (32 %). However, the non-sugar carbohydrate glucitol also demonstrated a dose-dependent inhibition of attachment (26 %).

DISCUSSION

The role of flagella and motility in the colonization of the infant mouse gut by *C. jejuni* has been investigated using two non-motile variants. These variants differed in the presence or absence of flagella and were selected from a clinical isolate of *C. jejuni*.

Although the selection did not involve the use of mutagens the possibility of other structural differences cannot be excluded. However, the only difference between the strains detectable by SDS-PAGE was the absence of a major 62 kDa and a minor 84 kDa protein from the outer membrane of the aflagellate variant. This 62 kDa protein has been shown to be flagellin (Newell, McBride & Pearson,

1984). The 84 kDa protein is possibly related to the 'cartwheel structure' which is found in close association with flagella and which has been shown to be absent in this aflagellate strain (SF-2) (Curry, Fox & Jones, 1984).

The flagella of the non-motile, flagellate variant (SF-1) and the wild-type strain had the same molecular weight by SDS-PAGE. A comparison of the amino acid composition of the two flagella preparations indicates that the proteins are similar. The differences, particularly in the glycine content, were probably attributable to minor contaminants. Moreover, both flagella preparations reacted identically with five monoclonal antibodies directed against campylobacter flagella (unpublished data). This data therefore indicates that the variants differed from the wild-type only in the absence of flagella and/or antibody.

Despite the relatively high molecular weight of campylobacter flagella (Newell, McBride & Pearson, 1984), their amino acid composition, in terms of mole percentage and relative proportions of hydrophobic, acidic, basic and aromatic residues, shows a marked consistency with previously published data for other bacterial flagella (Maruyama, Lodderstaedt & Schmidt, 1978).

The loss of serotype 6, by the aflagellate variant, in the slide agglutination technique indicates that flagella contribute significantly to the heat-labile serotyping scheme (Wenman *et al.* 1985).

In our investigations the wild-type strain (81116) colonized the gastrointestinal tract of the infant mouse in a very similar manner to that described previously for this (Newell *et al.* 1985) and other clinical strains of *C. jejuni* and *C. coli* (Field *et al.* 1981; Newell & Pearson, 1984). In this animal model intestinal epithelial invasion by campylobacters may occur (Newell & Pearson, 1984) but death or overt disease is rare (Field *et al.* 1981). Although the aflagellate variant (SF-2) colonized the infant mouse poorly, presumably because the organisms were rapidly eliminated from the gut, the non-motile, flagellate variant (SF-1) colonized the gut as successfully as, and occasionally better than, the wild-type strain. These results suggest that flagella, active or inactive, are necessary for the efficient colonization of the infant mouse gastrointestinal tract by *C. jejuni*.

Bacterial attachment to the intestinal mucosa is considered to be an important factor in colonization of the gastrointestinal tract (Giannella, 1981) and *in vitro* attachment studies may reflect the virulence of enteric pathogens. The attachment of *C. jejuni* to tissue culture cells *in vitro* was relatively poor; less than 2% in each experiment. Such levels of attachment are equivalent to 3–6 bacteria per cell. This rate of attachment compares favourably with the attachment of clinical strains of *C. jejuni* to tissue culture cells when observed by immunofluorescence (Newell *et al.* 1985). Although little difference was observed between the attachment of strain 81116 and strain SF-2, strain SF-1 attached significantly better than strain SF-2 or the wild-type strain to INT 407 and HeLa cells. These differences may be explained by the presence of an adhesin on flagella which gives the flagellated organisms an adhesive advantage over the non-flagellated organisms. However, the active flagella of strain 81116 presumably generate a tenuous association between the target cell and the motile organism whilst the inactive flagella of strain SF-1 would form a more permanent and efficient attachment. The increased attachment of strain SF-1 to the INT 407 cells, compared with the other epithelial cell lines, suggests the presence of a specific receptor for this

adhesin. As the cell line INT 407 was originally derived from human fetal intestinal epithelium (Henle & Deinhardt, 1957), this may reflect target tissue specificity.

Although all the sugars investigated inhibited the attachment of strain SF-1 to INT 407 cells, it is unlikely that the mechanism of interaction between the putative flagella adhesin and the target cell involves sugar receptors because the non-sugar carbohydrate glucitol caused a similar inhibition of attachment.

Previous studies using non-motile and motile variants of *V. cholerae* have demonstrated that motility was necessary for adherence to isolated brush borders and intact mucosal surfaces and for agglutination of red blood cells (Attridge & Rowley, 1983; Freter & Jones, 1976; Guentzel & Berry, 1975; Jones & Freter, 1976). Virulence was also dependent on the presence of motility (Attridge & Rowley, 1983; Guentzel & Berry, 1975; Yancy, Willis & Berry, 1978) and crude flagella vaccines were protective against experimental *V. cholerae* infections (Yancy, Willis & Berry, 1979). These studies suggest that flagella, or some factor associated with flagella, function to transport and attach *V. cholerae* to the intestinal mucosa and ensure efficient delivery of enterotoxin.

Our investigations indicate that the flagella of *C. jejuni* have similar adhesive properties and are likewise important in colonization. By the use of flagellate and aflagellate non-motile variants we have attempted to distinguish between motility and adherence. These investigations suggest that an adhesin, intimately associated with flagella, is involved in the virulence of *C. jejuni*. These studies do not eliminate the possibility of other adhesins on *C. jejuni*. Preliminary investigations using erythrocyte and buccal cell suspensions (unpublished data) indicate that other adhesins are expressed on the bacterial surface which are inhibited by the presence of active flagella. This is consistent with the distinct flagellum and somatic adhesins recently recognised on *V. cholerae* (Attridge & Rowley, 1983).

The identification of adhesins on pathogenic bacteria may allow the development of vaccines which will prevent attachment to mucosal surfaces (Watt, 1980). The flagella of *C. jejuni* induce a cross-reacting antibody response in humans during naturally acquired infections (Newell, 1983) and may therefore be a candidate component for use in a vaccine.

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