Campylobacter jejuni 81-176 forms distinct microcolonies on in vitro-infected human small intestinal tissue prior to biofilm formation

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INTRODUCTION

Campylobacter jejuni is the commonest cause of bacterial foodborne disease in the developed world and causes a large amount of disease, particularly in the young, in developing countries. Symptoms include bloody or watery diarrhoea, abdominal pain and fever (Skirrow & Blaser, 2000). It is evident that the organism must interact with host gastrointestinal epithelium in some way that manifests as the clinical presentation of diarrhoea (Konkel et al., 2001).

The interaction of C. jejuni with human tissue and how the organism causes diarrhoea in an infected host are not well understood. Experimental evidence using tissue culture cells as models of gastrointestinal epithelium suggests that bacteria interact with cells via their flagella. The flagellar export system delivers effector molecules into the host cell, which facilitates cell invasion (Konkel et al., 2001). CiaB has been shown to be important for the clinical presentation of diarrhoeal disease in a pig model of infection (Konkel et al., 2001). The organism may also bind to extracellular matrix molecules on the basolateral cell surface, which results in membrane ruffling and bacterial internalization (Konkel et al., 2001; Krause-Gruszczynska et al., 2007). Using C. jejuni-infected Caco-2 cells as a model of host cell infection, it is clear that bacterial adhesion to the apical cell surface is minimal, with most adhesion observed at cellular junctions (Hu et al., 2008). It is difficult to study the interaction of this organism with human tissue because biopsies are not always obtained from patients with C. jejuni enteritis due to the risk of intestinal perforation, or if a mucosal biopsy is taken at acute presentation the diagnosis of a specific Campylobacter aetiology may not be known. We have obtained human intestinal tissue (both ileal and colonic) from children undergoing elective intestinal resection and infected it in vitro with C. jejuni strain 81-176. This has allowed us to study the interaction of C. jejuni with human gastrointestinal tissue by electron and light microscopy.

METHODOLOGY

Bacteria. Bacteria were grown in Mueller–Hinton broth and agar (Oxoid) and incubated at 37 °C in a variable atmosphere incubator (VAIN, Don Whitley) under microaerophilic conditions in an atmosphere of 6% hydrogen, 5% carbon dioxide, 5% oxygen and 84% nitrogen. The strain of C. jejuni used in this study was 81-176 (+pVir). This strain has been shown to cause clinical disease in human volunteers (Black et al., 1988). Flagella mutants in flaA and flaB were also tested in both ileal and colonic tissue.
Infection of human intestinal tissue. Ethical permission to obtain and use human gastrointestinal tissue was sought and obtained from the Royal Hospital for Sick Children, research ethics committee, North Glasgow NHS Trust. Primary human small and large intestinal tissue was obtained from healthy sections of intestine removed during elective surgery. For this study at least 10 independent tissue samples were used from different patients. The tissue samples were initially transported to the laboratory in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS) and penicillin/streptomycin (Gibco). On arrival in the laboratory, tissue samples were washed in antibiotic-free DMEM with 10% FCS, cut into explants (1–2 cm²) and placed into the top well of a 12-well Transwell tissue culture plate (Corning). Explants were inoculated with 50 μl bacterial suspension (C. jejuni 81-176), containing ~1 × 10⁸ c.f.u., or left uninfected as controls. Explants were incubated for 1–4 h at 37°C in a 6% CO₂ humidified atmosphere to allow the bacteria to interact with the tissue. Uninfected control tissue from each different tissue source was used in the same experiments as infected tissue. Tissue was washed in three rinses of PBS prior to fixation and processing for microscopy. Tissue morphology was checked after infection by normal histological processing using haematoxylin and eosin staining. Only morphologically normal tissue was used for the study. Five independent experiments on ileal tissue were performed using flaA and flaB mutants of C. jejuni 81-176.

Specimen processing for scanning electron microscopy (SEM). Specimens were fixed for 1 h at room temperature in 2.5% glutaraldehyde in 0.1 M phosphate buffer. Following three 5 min rinses with 0.1 M phosphate buffer, specimens were fixed for 1 h in 1% osmium tetroxide. After three 10 min washes with distilled water, specimens were dehydrated through an ascending series of acetone solutions (30, 50, 70, 90%) twice for 10 min each, ending in 100% acetone twice for 10 min and dried 100% acetone twice for 10 min. Specimens were then dried in a critical point dryer (Polaron E3000) for 80 min and mounted on stubs using double-sided copper tape and silver paint. A Polaron SC515 SEM coating system was used to coat the specimens with gold–palladium (20 nm thickness) and they were viewed on a JEOL 6400 scanning electron microscope.

Specimen processing for immunohistochemistry. Human ileal sections of 4 μm thickness were mounted onto slides and allowed to dry before placing in a 37°C oven overnight. Slides were marked with

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**Fig. 1.** (a, b). Low-power views of C. jejuni 81-176 on ileal tissue, 1–2 h after infection. Organisms form microcolonies where mucus overlying intestinal villi is abundant. (c, d) Details of a microcolony. Bacteria adhere together in distinct clumps. Bacteria adhere to mucus via flagella and adhere to each other with a distinct interaction between flagella. (e) Detail of different microcolonies, showing distinct flagellar morphology.
the antibody and date, dewaxed in xylene and rinsed in 100 % alcohol then 95 % alcohol. Slides were then placed in 3 % hydrogen peroxide in methanol for 20 min at room temperature. After washing in water, antigen retrieval was performed by placing the slides in an autoclave for 10 min in citrate buffer, pH 6, at 121 °C. Slides were left to cool to 50 °C before the transfer of slides to a staining chamber. Slides were washed in PBS/Tween-20 twice before applying 25 % normal goat serum for 30 min at room temperature. Antibody against C. jejuni was added to the slides overnight at 4 °C (100 μl, 1 : 600 mouse monoclonal, BGN/2E10, 1 mg ml⁻¹, Abcam ab54125). A control isotype IgM antibody was used at equivalent dilution (Sigma M5909, 0.2 mg ml⁻¹). Slides were washed in PBS/Tween-20 twice before applying EnVision polymer (goat anti-mouse horseradish peroxidase conjugate, DakoCytomation) for 30 min. Slides were washed with PBS/Tween-20 twice before adding Vector NovaRED for 10 min, and again washed with water. Slides were counterstained with haematoxylin for 1 min, washed in water, dehydrated through alcohols then rinsed in xylene before mounting sections in DPX mountant.

RESULTS

In small intestinal tissue, bacteria were observed to adhere to mucus and cells via their flagella in distinct groups, as observed in Fig. 1. Organisms were particularly abundant on mucus overlying cells. Bacteria adhered to cells where mucus was absent but in much lower numbers or as solitary bacteria only. Separate groups formed on the mucosa distinctly adjacent to, but separate from, nearby groupings. Organisms initially, during early time points, did not coalesce over the entire surface of the mucosa and organisms were found to have formed distinct microcolonies on the tissue surface (Fig. 1–d). Bacteria in microcolonies adhered to the tissue via flagella. Bacterial aggregation in these groupings also occurred by flagellar interaction between individual organisms. Organisms adhered to each other and flagellar interaction within the microcolony was evident. Using mutants in $flaA$ and $flaB$ of C. jejuni 81-176 we were never able to show microcolony or biofilm formation in infected tissue. The flagella also exhibited a unique morphology (Fig. 1e), which was not evident in organisms grown in broth or suspended in tissue culture media alone (data not shown). These observations suggest microcolony formation of bacteria as an initial colonization step for the human mucosa. Microcolonies were particularly evident in small intestinal material, where the majority of these bacterial groupings occurred. There was a distinct difference in how organisms colonized the small and large intestinal tissue, with bacteria being more abundant in small bowel compared with colonic tissue. At later time points, organisms were observed as a biofilm over the surface of the ileal mucosa (Fig. 2). Immunohistochemical staining of human ileal tissue and viewing by light microscopy also showed a biofilm over the surface of the ileal tissue (Fig. 3).

In colonic tissue, bacteria were spread over the surface mucus, and distinct groups or microcolonies were not observed at up to 2 h post-challenge (Fig. 4). Occasional groups of organisms were observed in colonic crypts. Organisms were much more difficult to locate in the colonic tissue than in small intestinal tissue infected for the same time. Fig. 5 shows normal uninfected ileum and colon, demonstrating the normal morphology of these tissues at the end of the experimental time period (4 h).

Transmission electron microscopy (TEM) showed organisms interacting with surface mucus in close contact with cell microvilli (data not shown). Despite numerous attempts to locate large numbers of organisms by TEM, only small numbers were observed. It is not known whether the TEM processing affects organisms in tissue or removes most of the mucus overlying the epithelium.

**Fig. 2.** C. jejuni 81-176 forms distinct biofilms on human ileal tissue 3–4 h after infection *in vitro*. Bacteria are adherent to the mucosa, as tissue was washed before fixation.
tissue via flagella in discrete colonies or multicellular groups. In this study we have used a different strain of *C. jejuni* and larger tissue samples from human small and large intestine, and found differences in the bacterial distribution on tissues.

*C. jejuni* adheres to human small intestinal mucus in microcolonies via flagella and at later time points as a biofilm, shown as diffuse sheets of bacteria attached to human ileal tissue. Microcolony formation of this type is not observed in cell culture models of infection, which may reflect the lack of mucus in these model systems. Adhesion to small intestinal villus tip cells was also mediated by bacterial flagella (data not shown). The flagella have distinct morphology and mediate the adhesion of bacteria to each other within these microcolonies. The significance of the change in flagellar morphology is not known. Organisms within the same SEM field showed normal flagellar morphology, so only a subset of bacteria within the microcolony express this changed flagellar morphology. Microcolony or biofilm formation was never observed on ileal tissue when mutants in *flaA* or *flaB* were used. It has been shown that flagella are important for biofilm formation (Kalmokoff *et al.*, 2006; Guerry, 2007; Svensson *et al.*, 2008). At later time points, human tissue infection resulted in a distinct biofilm over the surface of the ileal tissue, a phenomenon observed *in vitro* with glass and plastic supports but as yet not observed in the organism’s interaction with tissue (Svensson *et al.*, 2008). The fact that the organisms appeared to cover the entire tissue surface raises questions as to how this observation may be linked to the organism’s ability to cause diarrhoeal disease in man. For many diarrhoeal pathogens colonization and persistence are important in the initiation of clinical symptoms. How *C. jejuni* causes diarrhoea in man is unknown in terms of the bacterial molecules that are required for the initiation of symptoms. Extensive colonization of the human gut mucosa would seem a reasonable pre-requisite for disease to occur. It is tempting to speculate that such a large concentration of organisms on an absorptive surface prevents the normal absorptive transport functions of the ileal mucosa and evokes other pathophysiological alterations.

It is clear from the published literature that *C. jejuni* can form biofilms (Svensson *et al.*, 2008; Hanning *et al.*, 2009; Asakura *et al.*, 2007). Published work relates to the formation of biofilms in the environment as a mechanism of persistence and possible transmission and survival within the poultry house (Howard *et al.*, 2009; Svensson *et al.*, 2009). Water in poultry houses has been shown to be a possible source of infection for birds and it has also been shown that *C. jejuni* is present in biofilms in drinking apparatus (Hanning *et al.*, 2008). The biofilm consists of aggregates and planktonic bacteria that live in a community consisting of either a single species or, when present in the environment, multiple species. Presumably this way of life confers some survival benefit on the members of the community. The organisms within the biofilm show

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**Fig. 3.** Immunohistochemistry of human ileal tissue *in vitro*-infected with *C. jejuni* 81-176 (3–4 h after infection). The red staining is specific for *C. jejuni*. No staining was observed for uninfected tissue (not shown). The organism forms a biofilm on human ileal tissue. Bar, 500 μm.

**DISCUSSION**

In similar work by Grant *et al.* (2006), using smaller endoscopic biopsies of duodenum and then infecting *in vitro*, *C. jejuni* 11168 was shown to adhere to mucosal
changes in metabolic activity and cell shape, and increased tolerance to various stresses including enhanced resistance to antimicrobial agents (reviewed by Svensson et al., 2008). Although microcolony formation has been shown to be important for biofilm formation *in vitro* (Guerry, 2007; Golden & Acheson, 2002), it has not been shown before that biofilm formation occurs on tissue *in vivo* or using an *in vitro* tissue infection model.

Bacteria were more readily visualized in ileal tissue than in colonic tissue, and whether this reflects the true tissue distribution in human infection is unknown and merits further study. This is perhaps surprising in view of the dogma that *C. jejuni* infection is predominantly a colitis, with major colonic involvement in disease (Skirrow & Blaser, 2000). Our observations raise the possibility that the infection starts in the ileum, with more severe disease extending to the colon, as observed in salmonellosis (Skirrow & Blaser, 2000). Such a scenario is supported by earlier observations of ileal involvement in occasional patients who had undergone laparotomy or were examined post-mortem (Butzler, 1981).

Human intestinal tissue obtained at operation is a valuable resource for studying the interaction of *C. jejuni* with its target tissues in an infected host, and has shown the formation of multicellular communities on tissues to be a consistent feature of different strains of *C. jejuni* (Grant et al., 2006). These models should facilitate the study of the interaction of the bacterium with its target tissue at a level of complexity not possible except in animal models, and could be used to help identify disease-causing mechanisms of *C. jejuni*.

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