Campylobacter jejuni adhere to and invade chicken intestinal epithelial cells *in vitro*

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Campylobacter jejuni is a common cause of serious diarrhoeal disease in humans, in contrast to the avian population, where exposure results in prolonged colonization at high density without disease. Colonized poultry present a significant source of infection to humans worldwide. The aim of this work was to compare the interaction of *Campylobacter* with primary intestinal cells from humans and poultry to identify factors that account for the divergent outcome following *Campylobacter* exposure. A primary intestinal cell model of *Campylobacter* infection was developed using cells grown from human and chicken intestinal biopsies. The cultured cells were infected with a number of strains of *Campylobacter*. Invasion by *C. jejuni* and the influence of intestinal mucus on *Campylobacter* internalization were studied by fluorescence microscopy and gentamicin protection assays. *C. jejuni* invaded primary human intestinal cells in a microtubule-, microfilament- and caveolin-dependent manner. Entry of *C. jejuni* into primary chicken intestinal cells also occurred. Chicken mucus, but not intestinal mucus of human origin, significantly reduced infection of primary human intestinal cells. Avian mucus appears to inhibit *Campylobacter* from interacting with epithelial cell surfaces.

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INTRODUCTION

The bacterial microflora of the gastrointestinal tract has diverse effects on the host in both health and disease (Falk *et al.*, 1998; Guarner & Malagelada, 2003). Although the faecal microbiota conventionally is viewed as having a symbiotic relationship with the host, when viewed individually many members of the faecal flora are better described as commensals or opportunists [i.e. existing for their own benefit, but without conferring beneficial (symbiotic) or detrimental (pathogenic) effects on the host (Falk *et al.*, 1998)]. It is well recognized that some commensal enteric organisms become pathogenic under specific conditions (Falk *et al.*, 1998; Farthing, 2004) (e.g. *Clostridium difficile*) and that the pathogenic effect of some enteric organisms is potentiated in a restricted host range or age group (e.g. *Escherichia coli* O157:H7, *Salmonella* and *Campylobacter*) (Moxley, 2004; Rabsch *et al.*, 2002).

Campylobacter are the principal bacterial cause of food-borne gastrointestinal disease in developed countries and probably also in the developing world (Blaser, 1997; Crushell *et al.*, 2004; Takkinen *et al.*, 2003). Additionally, *Campylobacter* can cause invasive disease and extra-intestinal sequelae including bacteraemia and Guillain-Barré syndrome (Hannu *et al.*, 2002; Reed *et al.*, 1996).

Epidemiological studies identify poultry to be a major reservoir of human infection (Frost, 2001; Hanninen *et al.*, 2000; Nadeau *et al.*, 2003). Chickens are commonly colonized by *Campylobacter jejuni*, with some studies showing up to 98 % of point-of-sale product contaminated (Hanninen *et al.*, 2000; Jacobs-Reitsma, 2000; Madden *et al.*, 1998; Pearson *et al.*, 2000). Despite the high prevalence of *C. jejuni* in chickens, intestinal disease does not appear to occur following naturally acquired infection, avian intestinal inflammation is absent, and no cellular attachment or invasion is demonstrated in the intestine of colonized birds (Beery *et al.*, 1988; Meinersmann *et al.*, 1991).

Therefore, although pathogenic to humans, *C. jejuni* behaves as a commensal in avian hosts. Knowledge of the mechanisms underlying these differences in disease outcome following *Campylobacter* exposure could serve as a useful model for understanding pathogen–commensal relationships in the gastrointestinal tract, and might provide a better understanding of how *Campylobacter* colonizes hosts and causes enteric disease. We speculated that failure of *C. jejuni* to attach to or invade avian intestinal cells *in vivo* (Beery *et al.*, 1988; Meinersmann *et al.*, 1991) reflects a species-specific tissue tropism. To explore this hypothesis we used a primary intestinal cell model to examine the interaction of *C. jejuni* with a variety of host intestinal cells *ex vivo*. *C. jejuni* readily invaded primary human and avian intestinal epithelial cells. We demonstrate that chicken-derived intestinal mucus, but not human mucus, attenuated

Abbreviations: FCS, fetal calf serum; TAMRA, carboxytetramethyl-rhodamine.

C. jejuni invasion *in vitro*. From these data we conclude that specific avian intestinal luminal factor(s) rather than tissue tropism underlie *Campylobacter* commensalism in chickens. These findings provide an important basis for further exploration of the effect of mucin and other constituents of the mucus layer on bacterial pathogenicity during enteric infection.

METHODS

Bacterial strains and culture conditions. The following strains were used in this study: *C. jejuni* 81-176, a well-characterized strain widely used in pathogenesis studies (Kopecko *et al.*, 2001); *C. jejuni* NCTC 11168, the first sequenced strain (Parkhill *et al.*, 2000); *C. jejuni* F1882 and F1998, low-passage human isolates (Byrne *et al.*, 2001); and CB123, a fresh human isolate. *C. jejuni* 1646DF and 1449BF were fresh chicken isolates. Organisms, including most clinical isolates, were stored at -70°C in 10% (v/v) glycerol, 10% (v/v) fetal calf serum (FCS), 80% (v/v) Brucella broth. They were recovered on Columbia agar (Oxoid) containing 7% (v/v) defibrinated horse blood, incubated at 37°C under microaerophilic conditions generated by Campygen gas packs (Oxoid). *C. jejuni* CB123 was freshly isolated on CCDA selective medium (Oxoid) and plated onto Columbia blood agar. Replicate experiments were conducted within two to four subcultures of primary isolation over a time frame of a week. For infection assays *Campylobacter* isolates were grown in a biphasic medium for approximately 18 h. Biphasic medium was prepared using a Columbia agar slant overlaid with 7 ml cell culture medium in 25 cm² culture flasks. Enumeration of bacteria following invasion assays was achieved by dilution in phosphate-buffered saline (PBS) and plating onto *Campylobacter* blood-free selective agar base (Oxoid CM739), without selective supplement but with an additional 0.1% (w/v) agar to minimize the swarming of mucoid isolates. Clinical isolates were identified using an API Campy system (bioMérieux). *E. coli* HB101, a non-invasive control organism, was grown on Columbia blood agar at 37°C in air for 18–24 h. Post-infection assays were plated to MacConkey agar.

Primary cell isolation and culture. Biopsy material was obtained from children undergoing endoscopy at Our Lady's Children's Hospital, Dublin, Ireland. Ethical permission for this study was obtained from the ethics committee of the hospital. Informed, signed consent was obtained from parents/guardians of all children enrolled in the study. Mucosal biopsy specimens were taken from the first part of the duodenum using a standard endoscopic biopsy forceps. Only grossly normal tissue was sampled. Chickens (Cobb 500, commercial broiler) aged between 6 and 12 weeks were killed by injection of Euthatal (Merial Animal Health). Sections of distal small intestine were excised, slit longitudinally, fixed in position and rinsed in cold PBS. Biopsies were taken using endoscopy forceps and placed into primary cell culture medium at 4°C for transport. Cells were isolated essentially as described by Clyne & Drumm (1993) for isolating human gastric cells. Briefly, biopsies were incubated in 15 ml Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} or Mg^{2+} , containing 0.1 mM EDTA and 0.1 mM DTT, with vigorous shaking for 15 min at 37°C . Collagenase, 0.05% (w/v) (Sigma) in RPMI, was applied to the biopsies, which were reincubated with shaking for periods of 10 min until digestion was complete. The supernatant containing released cells and crypt fragments was aspirated to a tube containing 5 ml primary cell medium (see below) containing 10% (v/v) FCS to halt digestion. The suspension was centrifuged at 800 g for 5 min at 4°C . Cellular harvests were suspended in fresh medium and held on ice until further digestion of biopsies was complete. The combined harvests were again washed in primary medium and resuspended in a small volume (2–4 ml) of

medium. The harvested crypts were examined using an inverted microscope and seeded onto 13 mm plastic Thermanox coverslips (Nunc, Thermanox 174950) in 24-well trays. Harvested primary human and chicken intestinal cells were grown in medium comprising 50:50 DMEM-F12, 10% (v/v) FCS, 8 μg insulin ml⁻¹, 50 μg hydrocortisone ml⁻¹ (Sigma), 10 μg gentamicin ml⁻¹, 2.5 μg amphotericin ml⁻¹, 100 μg penicillin ml⁻¹ and 100 U streptomycin ml⁻¹ (BioWittaker). The medium was supplemented at 18 h without disturbing the attaching cells and culture continued. Preparations were used approximately 42–48 h post-seeding. Except where stated, all cell cultures were grown at 37°C in a humidified atmosphere containing 5% CO_2 . Chicken epithelial harvests were initially cultured at both 37°C and 42°C . Prior to assays, cells were rinsed twice in pre-warmed antibiotic-free primary medium to remove non-adherent cells and reincubated in antibiotic-free medium for a minimum of 2 h prior to infection.

Collection of mucus. A sterile universal container was used to scrape gently along the surface of the excised chicken intestine in order to collect crude intestinal mucus. This was then emulsified with ~15% (v/v) PBS to facilitate mixing and dispensing and stored at -20°C . Crude human mucus, obtained in a similar manner from human intestinal specimens, was a generous gift from Professor Prem Puri, The Children's Research Centre, Dublin. Mucus was confirmed free of enteropathogens, including *Salmonella*, *Shigella* and *Campylobacter*, using culture to appropriate medium. Control experiments verified that the mucus used in these experiments did not exhibit antibacterial activity against *C. jejuni* 81-176. Attendant normal microflora was present.

Antibodies and staining. *C. jejuni* polyclonal antiserum was raised in rabbits. *C. jejuni* 81-176, 11168 and F1882 were harvested from agar plates. The suspensions were pooled and washed in PBS. Bacteria were suspended in PBS containing 0.5% (v/v) formaldehyde at a concentration of 1 g (wet wt) per 100 ml of suspension. The immunization protocol was as previously described for the generation of *Helicobacter pylori* antiserum (Clyne & Drumm, 1993). Vital staining (red) of bacteria was achieved by incubating organisms in PBS containing 10 μg carboxytetramethylrhodamine ml⁻¹ (TAMRA T-6105, Molecular Probes) for 30 min at 37°C in the dark, followed by washing four times in PBS (Mooney *et al.*, 2003).

Cell cultures were fixed using methanol or 4% formaldehyde in PBS as appropriate. Where required, cells were permeabilized using 0.1% (v/v) Triton X-100 (Sigma). Anti-pancytokeratin (Sigma C-2562) revealed by anti-mouse FITC (Sigma F8771) was used to confirm the epithelial origin of human intestinal cells (Mooney *et al.*, 2003). An FITC-conjugated cytokeratin antibody (Sigma F 0397), which includes chicken in the species range, was used to stain chicken intestinal cells. Methanol-fixed cells were stained with Giemsa (BDH) and mounted in DPX. Primary cells, invaded with red-labelled *C. jejuni*, were fixed and stained as follows. Preparations were blocked with 10% (v/v) goat serum in PBS, incubated in *C. jejuni* antiserum, 1:200 in bovine serum albumin, washed, and incubated in 1:200 goat-anti-rabbit IgG Alexa Fluor 488 (Molecular Probes A-11070), labelling external organisms green. Cells were then permeabilized using 0.1% (v/v) Triton X-100 for 4 min and stained with 1:6000 TRITC-phalloidin (Sigma P1951) or 1:2000 FITC-phalloidin (Sigma P 5282) for 6 min, permitting clear discrimination between organisms and cellular morphology. Nuclei were visualized by including DAPI (Sigma) at a dilution of 1:4000. Stained preparations were mounted in fluorescent mounting medium (Dako) and sealed with clear nail polish.

Adherence assays. Cell culture medium was replaced with 4×10^7 TAMRA-labelled *E. coli* or *C. jejuni* (strains listed above) in 500 μl antibiotic-free medium containing 1% (v/v) FCS and centrifuged at 250 g for 5 min at room temperature. Trays were incubated at 37°C

for 2 h in a microaerophilic environment unless otherwise stated. At the end of incubation, coverslips were washed four times in PBS, fixed and stained.

Invasion assays. Microscopic discrimination of internalization into primary human and primary chicken cells was established by differential staining of adherent organisms. Following infection assays, TAMRA-labelled adherent organisms (red) were stained green using a specific *C. jejuni* antibody revealed by a green probe (Alexa Fluor 488), leaving the internalized organisms single stained (red) while adherent organisms were double labelled (red and green). In addition to growing and infecting cells at 37 °C, primary chicken cells also were grown and infected at 42 °C using TAMRA-labelled organisms. Additionally, gentamicin protection was used to evaluate internalization of *Campylobacter* into primary human and chicken cells. These invasion assays were set up as for adherence, omitting the bacteria-labelling step. The invasion of a number of strains was examined at the same organism density, under the same conditions. At the end of the invasion period, cells were washed in PBS to remove non-adherent organisms and reincubated in medium containing 400 µg gentamicin ml⁻¹ (BioWittaker) for a further 2 h to kill adherent organisms (Mooney *et al.*, 2003). Cells were washed in PBS, and incubated in 200 µl PBS with 0.1 % (v/v) Triton X-100 for 15 min at 37 °C to release intracellular organisms. Dilutions of replicate wells in PBS were plated and colonies enumerated after 48 h incubation. Primary cells grew as discontinuous islets at ~10⁴–10⁵ per well. As primary cell numbers are substantially lower than those conventionally employed, results are expressed as c.f.u. per well rather than percentage inoculum internalized.

Effect of inhibitors on primary cell invasion. The effect of cytochalasin B (Sigma) (inhibitor of actin polymerization), vincristine (Sigma) (inhibitor of microtubule polymerization) and filipin (Sigma) (caveolin inhibitor) on internalization of *C. jejuni* 81-176 into primary human intestinal cells was assessed using the gentamicin protection assay. Inhibitors were added to primary cells 1 h prior to the addition of bacteria and maintained throughout the assay. Control experiments verified that inhibitors did not affect cell viability using trypan blue exclusion or bacterial viability by plating experiments.

Primary cell invasion assays in the presence of mucus. The effect of various mucus preparations on internalization of *C. jejuni* 81-176 into primary human intestinal cells was examined. Primary intestinal cells were challenged using 200 µl *C. jejuni* suspended in medium at a concentration of 8 × 10⁷ c.f.u. ml⁻¹ (control) or in medium containing different mucus preparations at ~15–20 % (v/v). Preparations containing organisms and mucus were mixed briefly using a vortex and applied to cultured cells. Trays were centrifuged at 250 g for 5 min at room temperature and incubated at 37 °C in microaerophilic conditions for 2 h. Internalization was measured using gentamicin protection.

Effect of mucus concentration on total cell association. Given the relatively restricted availability of primary human intestinal cells, the concentration of chicken mucus required to reduce *Campylobacter* cell association was examined using confluent Caco-2 cells grown in 96-well trays. Cells were seeded at 2.5 × 10⁴ per well and cultured for 11 days in Eagle's Minimal Essential Medium (EMEM) (Bio-Whittaker) with 10 % (v/v) FCS, 1 % (v/v) non-essential amino acids and 2 mM L-glutamine. Chicken small intestinal mucus was dispensed into sterile tubes to give a range of concentrations starting at 1 % (w/v). Some mucus preparations were liquefied using a DTT-containing agent (Sputasol, Oxoid SR0233A). *C. jejuni* 81-176 was added to each preparation to a final concentration of 8 × 10⁷ c.f.u. ml⁻¹. Cells were challenged by *C. jejuni* in medium or in chicken mucus-containing medium in 100 µl volumes

and incubated for 2 h at 37 °C in a microaerophilic atmosphere. Mucus-containing supernatants were aspirated carefully and the cells washed four times in PBS. Intracellular organisms were released by incubation in 100 µl PBS with 0.1 % (v/v) Triton X-100 for 15 min. Total cell-associated organisms were enumerated by plating.

Imaging. A Nikon TMS inverted microscope and Olympus camera were used to examine and record patterns of primary cell growth. Stained slides were examined using blue, green and red filters with a Leica DML fluorescent microscope. Images were acquired using a Leica DC camera and manipulated using Adobe Photoshop 6. Confocal images were acquired using a Zeiss LSM UV 510 META confocal microscope. Merged images show internal organisms in red, external organisms in yellow (i.e. combined red and green) and nuclei in blue. Cellular actin is stained faintly in red in some images. XYZ series were taken through areas of interest.

Statistical analysis. Experiments were performed on three or more occasions using cells harvested from different patients or chickens. Graphs were drawn using Microsoft Excel. Data from experiments were analysed using Minitab. Statistical significance was estimated using the non-parametric Mann–Whitney test when comparing primary cell infections or two-tailed Student's *t* test when examining the influence of mucus concentration. In all cases differences were considered significant at *P* ≤ 0.05.

RESULTS

Primary cell culture and morphological characteristics

Islets of cobblestone cells consistent with an epithelial lineage were cultured from both human and chicken intestinal tissue (Fig. 1a, b). They could be seen to emanate from harvested intestinal crypts, and some coalesced to yield areas of confluent growth. Primary human intestinal cells were maintained in culture for up to 3 weeks. Cytokeratin staining was used to confirm epithelial lineage. Initial chicken harvests were cultured at both 37 °C and 42 °C and maintained for up to 10 days. No difference was noted in extent of growth, appearance, size of cells or duration in culture. Thereafter, chicken cells were cultured at 37 °C only. Chicken cells were marginally smaller than human-derived cells. In addition to islets of epithelial lineage, chicken biopsies initially yielded small numbers of fibroblasts and macrophages (rarely observed in human cultures). Contamination with non-epithelial cell lineages was reduced by decreasing the biopsy depth (to avoid sampling the mucosa of the thinner avian intestine) and by allowing crypt fragments to sediment naturally in a conical tube for ~5–7 min, removing the supernatant, containing isolated cells and using the crypt-enriched deposit to seed wells.

Attachment to and invasion of primary human intestinal cells by *C. jejuni*

TAMRA-labelled *C. jejuni* (red) readily associated with and invaded cultured human intestinal epithelial cells. Internal organisms (labelled red only) were distinguished from adherent organisms (labelled red and antibody stained with green) by switching the filters on the fluorescent

Fig. 1. (a) Cultured primary human intestinal cells, after ~48 h in culture on Thermanox coverslips showing two islets emanating from crypt epithelium (arrowheads) beginning to coalesce and generate areas of confluence. Very occasional fibroblasts were recovered (arrow). (b) An islet of primary chicken cells at ~48 h in culture demonstrating a dense colony emanating from a fragment of crypt epithelium (arrowhead). Images of live cell cultures were taken using an inverted microscope. Bars, 50 μ m.

microscope. Confocal images of a merged Z series through parts of a colony of infected primary human cells show internalized *C. jejuni* in red and adherent *C. jejuni* in yellow (i.e. merged red and green). The pattern of cellular association of adherent and internalized organisms was markedly uneven (Fig. 2a, b, c). In contrast to *C. jejuni*, TAMRA-labelled *E. coli* HB101 did not associate with primary human intestinal cells.

The gentamicin protection assay was employed to quantitatively assess internalization by a number of different strains. Internalization data are presented as c.f.u per well recovered following gentamicin protection. Comparisons between different strains, or individual strains under different conditions, were performed using cells derived from the same patient(s) to minimize variability.

As shown in previous studies (Biswas *et al.*, 2000; Konkel *et al.*, 1992; Schwartz *et al.*, 1996; Tay *et al.*, 1996) using conventional tissue culture, levels of invasiveness varied considerably among strains. Invasion of primary human intestinal cells using a variety of *C. jejuni* isolates is shown in Table 1. With one exception (F1889), fresh isolates from both humans and chickens tended to invade more efficiently than *C. jejuni* 81-176. All differences were significant at $P < 0.05$ compared to *C. jejuni* 81-176 ($n = 6$).

In order to further characterize this primary cell model of *C. jejuni* infection, we evaluated the effects, on cell entry, of various inhibitors of cellular processes known to interfere with *Campylobacter* infection of conventional tissue culture cells. Specifically, the effect on invasion of vincristine, cytochalasin B and filipin, inhibitors of microtubule polymerization, actin polymerization and sterol-binding disruption of caveolae and caveola-like structures, was compared to controls (no inhibitor), using gentamicin protection. Viability of cells or organisms was not affected in the presence of inhibitors. All inhibitors markedly reduced primary cell invasion by *C. jejuni* 81-176. Preincubation of cells with vincristine ($1 \mu\text{g ml}^{-1}$) cytochalasin B ($10 \mu\text{g ml}^{-1}$) and filipin ($5 \mu\text{g ml}^{-1}$) (inhibitors of microtubulin, actin and caveolin, respectively) resulted in a mean $\pm$ SD percentage recovery of $20 \pm 11\%$, $15 \pm 15\%$ and $5 \pm 6\%$ of invasion control, respectively ($n = 9$). All decreases were statistically significant compared to untreated cells ($P < 0.05$).

Attachment to and invasion of primary chicken intestinal cells by *C. jejuni*

As *Campylobacter* does not attach to or invade chicken epithelial cells *in vivo* (Beery *et al.*, 1988; Meinersmann *et al.*, 1991), we hypothesized that *C. jejuni* would not invade primary avian intestinal cells *in vitro*. Primary chicken intestinal cells were challenged with a number of different TAMRA-labelled strains of *C. jejuni*. Because avian intestinal core temperature ranges between 41.5 and 42 °C, preliminary cell cultures and invasions with TAMRA-labelled *C. jejuni* were conducted at both 37 °C and 42 °C. Microscopic examination showed no detectable difference in cell growth or bacterial attachment and entry at these two temperatures (data not presented); therefore subsequent incubations and assays were conducted at 37 °C. Microscopic examination clearly showed that *C. jejuni* attached to and entered cultured primary chicken intestinal cells (Fig. 2d). The extent and pattern of *C. jejuni* invasion of chicken intestinal cells were comparable to those observed with primary human cells (Fig. 2a, b, c). *E. coli* HB101 did not associate with chicken epithelial cells to any significant extent.

Similar to invasion of primary human intestinal cells, invasiveness varied between *C. jejuni* isolates; in general, fresh isolates showed slightly higher rates of invasion than *C. jejuni* 81-176 (Table 1; $n = 6$). Apart from the chicken isolate 1646DF, the differences were significant ($P < 0.05$) compared to *C. jejuni* 81-176.

Effect of mucus on *Campylobacter* internalization into primary intestinal cells

Efficient internalization of *C. jejuni* into primary chicken intestinal cells was unexpected given its lack of association with chicken crypt epithelium *in vivo*. Therefore, we postulated that the failure of bacteria to associate with cells *in vivo* might result from avian luminal factor(s) that differ from those of humans. Colonization of the enteric mucus layer is a key early step in enteric pathogenesis. As avian mucus is readily and persistently colonized by *C. jejuni* (Stern *et al.*, 1988) (without cellular association) we explored the influence of human- and chicken-derived mucus on primary human intestinal cell invasiveness. As the relative colonization of the proximal small intestine by *C. jejuni* is lower than that of the large intestine in chickens (Beery *et al.*, 1988;

Fig. 2. (a, b) Confocal images (part of a Z series) of cultured primary human intestinal cells infected with TAMRA (red)-labelled *C. jejuni* 81-176. A specific *C. jejuni* antibody conjugated to Alexa Fluor 488 (green) was used to stain adherent organisms, now yellow/green. Nuclei are stained blue with DAPI. These two fields from the same invasion illustrate typical patterns of organism distribution and internalization at the periphery of a confluent islet, where cell association and internalization are high. (c, d) Confocal images (part of a Z series) of an area of (c) primary human intestinal cells and (d) primary chicken intestinal cells, showing mainly internalized TAMRA-labelled *C. jejuni* 81-176, with occasional adherent organisms (arrows).

Hendrixson & DiRita, 2004), the effects of small and large intestinal mucus were examined. Control experiments showed that neither organism nor cell viability was affected in the presence of mucus under the experimental conditions. Infection of primary cells in the presence of intestinal mucus modified internalization, but its effect differed depending on the source of the mucus. Crude human mucus increased internalization of *C. jejuni* by $35 \pm 22\%$ ($P=0.61$). In contrast, chicken large intestinal mucus reduced *C. jejuni* entry into primary human intestinal cells by $54 \pm 18\%$ and chicken small intestinal mucus reduced entry by $98 \pm 2\%$ ($P=0.0350$ and 0.0000 respectively).

Effect of mucus concentration on total cell association

To explore in more detail the influence of chicken mucus on the ability of *Campylobacter* to infect intestinal cells,

infection assays were carried out over a range of mucus concentrations. Chicken small intestinal mucus reduced cell association of *C. jejuni* with Caco-2 cells in a dose-dependent manner (Fig. 3). As little as 2% chicken mucus significantly reduced *Campylobacter* recovery in these infection assays ($P<0.05$). Interestingly, liquefaction of chicken mucus using Sputasol did not abrogate the inhibitory effect of mucus on cell association.

DISCUSSION

In contrast to the acute enterocolitis typical of human *C. jejuni* infection, poultry exposed to *Campylobacter* exhibit prolonged colonization without evidence of pathological change in the intestine (Beery *et al.*, 1988; Meinersmann *et al.*, 1991). Although immature birds challenged by large *C. jejuni* inocula may get diarrhoea and some reports allude to

Table 1. *C. jejuni* invasion of primary human and chicken intestinal cells

Values represent number of c.f.u. per well recovered following 2 h invasion assay of either human or chicken primary intestinal cells using the gentamicin protection assay (*n*=6).

Strain	C.f.u. per well	
	Human intestinal cells	Chicken intestinal cells
Human isolates		
81-176	$(7.97 \pm 0.8) \times 10^2$	$(1.05 \pm 0.65) \times 10^3$
F1882	$*(3.02 \pm 0.9) \times 10^3$	$*(2.61 \pm 0.49) \times 10^3$
F1889	$*(1.85 \pm 0.64) \times 10^2$	$*(1.92 \pm 1.1) \times 10^2$
CB123	$*(3.715 \pm 1.4) \times 10^3$	$*(2.52 \pm 1.06) \times 10^3$
Chicken isolates		
1646DF	$*(1.96 \pm 0.24) \times 10^4$	$(1.46 \pm 1.3) \times 10^3$
1449BF	$*(1.51 \pm 0.26) \times 10^3$	$*(2.76 \pm 0.4) \times 10^3$
<i>E. coli</i>		
HB101	$(4.9 \pm 6) \times 10^1$	†

*Denotes a statistically significant difference (*P*<0.05) compared to *C. jejuni* 81-176.

†*E. coli* HB101 was found to enter cultured avian cell monolayers in some experiments. Subsequent assays and microscopic examinations revealed organisms within contaminating macrophages and not in epithelial cells.

a form of vibronic hepatitis, there is no evidence that naturally acquired *C. jejuni* causes infection in avian intestinal epithelium cells (Newell, 2000; Skirrow, 1994). Recently, Smith *et al.* (2005) used an avian kidney cell model of *Campylobacter* infection and showed adherence to and invasion of these cells but the interaction of *Campylobacter* with avian intestinal cells has never been studied directly. In this study we have developed a primary cell model of intestinal campylobacteriosis to investigate the host-specific tropism of these organisms. Contrary to expectation, *C. jejuni* efficiently adhered to and entered avian intestinal epithelium. Chicken (but not human) intestinal mucus effectively attenuated *C. jejuni* invasiveness and reduced cell association in a dose-dependent manner, suggesting that a component of chicken mucus may be responsible for the divergent disease outcomes in humans and poultry following *Campylobacter* exposure.

In previous studies of *Helicobacter* it has been shown that primary cells obtained from human endoscopic biopsy samples provide a biologically relevant model of gastro-intestinal infection that may reflect more precisely the intestinal milieu than conventional tissue culture (Clyne & Drumm, 1993; Clyne & Drumm, 1997). In a previous report we showed that *Campylobacter upsaliensis* adheres to and invades primary intestinal cells (Mooney *et al.*, 2003). More recently, primary cells have been used to model the tissue tropism seen *in vivo* with *Cryptosporidium* species (Hashim

Fig. 3. Chicken mucus modifies *C. jejuni* 81-176 association with Caco-2 cells in a concentration-dependent manner. Cells were infected with *C. jejuni* 81-176 in culture medium alone (M) or in medium containing chicken mucus 1–10% (w/v) or with chicken mucus 10% (w/v) liquefied by DDT (10%-DDT). DDT did not affect organism viability. Values represent the mean c.f.u. recovered $\pm$ SD of three replicate infections. *Denotes a statistically significant difference (*P*<0.05) compared to infection in medium alone.

et al., 2004). In this study we have further explored this *ex vivo* model of enteric infection. We have shown that primary human intestinal cells remain viable for prolonged periods and are not overgrown with non-epithelial lineages. Using inhibitors of cellular processes previously described to affect *Campylobacter* pathogenicity in immortalized cell lines, we have confirmed the importance of host microtubules, microfilaments and lipid rafts for cellular uptake of these organisms.

Some strains of *C. jejuni*, most notably the well characterized 81-176 isolate, previously have been described as being dependent on microtubule polymerization, but independent of actin, for effective entry into epithelial cells (Hu & Kopecko, 1999; Oelschlaeger *et al.*, 1993). In contrast, other investigators have shown that microfilament inhibitors effectively reduce host cell entry by *C. jejuni* isolates and other campylobacters (Biswas *et al.*, 2000; Mooney *et al.*, 2003). In the present study, *C. jejuni* 81-176 was inhibited from invading primary cells previously exposed to inhibitors of actin accumulation. These results are in keeping with those recently reported for *C. jejuni* invasion of INT 407 cells by Monteville *et al.* (2003). The contribution to cell entry of a variety of different cell processes, including lipid rafts and cytoskeleton components, is an emerging paradigm among enteric pathogens (Cossart & Sansonetti, 2004). The use of primary cells can provide important information regarding the role *in vivo* of proposed pathogenic mechanisms without necessitating animal or human challenge. Primary cell models also serve to validate the performance of existing cell line models (Pageot *et al.*, 2000). We believe that they will have an important role in future exploration of the cellular microbiology of *Campylobacter* and other enteric pathogens.

Given the evidence for lack of interaction between *C. jejuni* and avian epithelium *in vivo* (Beery *et al.*, 1988;

Meinersmann *et al.*, 1991), it was surprising that a number of *C. jejuni* isolates (of both human and poultry origin) invaded chicken cells with an efficiency that was comparable to invasion of primary human intestinal cells *in vitro*. Clearly, then, the lack of chicken intestinal epithelial cell association *in vivo* does not reflect absence on these cells of the appropriate receptor-ligand(s) to effect *Campylobacter* adhesion and invasion. Our experiments suggested that the temperature differences that exist between humans and chickens (37 °C versus 42 °C) did not influence significantly the pathogenicity of the organisms (data not shown). In common with intestinal commensals in general, *C. jejuni* colonizes the intestinal mucus layer in chickens (Beery *et al.*, 1988; Meinersmann *et al.*, 1991) and components of mucus act as chemoattractants (Hugdahl *et al.*, 1988). Therefore, we examined the relative effects of intestinal mucus derived from humans and poultry. Chicken mucus markedly reduced invasion of primary human cells, without affecting organism viability. In contrast, human mucus tended to promote *C. jejuni* invasion although the results did not achieve statistical significance ($P=0.61$).

The nature of the relationship between the host and its faecal microflora and the role of this relationship in health and disease currently is the focus of intense research. The present view is that the intestinal microbiota are maintained in a dynamic relationship with the host involving a complex 'dialogue' between micro-organism, epithelium and mucosal immune system (Bourlioux *et al.*, 2003; Falk *et al.*, 1998). Recent work has started to uncover some important bacterial factors necessary for successful avian colonization by *C. jejuni* (Hendrixson & DiRita, 2004; Jones *et al.*, 2004; Karlyshev *et al.*, 2004; Nachamkin *et al.*, 1993). However, the specific host factors responsible for establishing and maintaining the affiliation of micro-organisms with the intestinal environment remain largely unknown. Species differences in mucus or associated factors may be sufficient to transform the behaviour of a pathogen in humans to commensal behaviour in another species. This would have substantial implications for furthering our understanding of the ecology of the intestinal tract.

At present it is not clear what component(s) of mucus that differ(s) between humans and poultry attenuated *Campylobacter* virulence in this study. An early study of intestinal mucus by McSweeney *et al.* (1987) suggested a role for rabbit secretory IgA in the blocking of *Campylobacter* adherence to INT 407 cells. Recently, it was shown that avian kidney cells and macrophages express pro-inflammatory cytokines and chemokines in response to *Campylobacter* infection (Smith *et al.*, 2005). These studies raise the possibility that the avian immune system could influence *Campylobacter* infectivity *in vivo*. However, the chickens used in our experiments were housed in bio-secure facilities and were confirmed not to harbour *Campylobacter* before use. Furthermore, although chicken mucus attenuated virulence in our experiments, no effect on bacterial viability was noted. Therefore, we feel it is unlikely that the observed

reduction in bacterial virulence, in our experiments, following exposure to mucus was caused by a specific avian immune response or antibacterial metabolites.

The pH of the chicken intestinal tract is lower (pH 5.7–6.4) (Denbow, 2000) than that of humans, a factor that potentially could affect virulence. However, the mucus used in these experiments was buffered to a neutral pH, and therefore increased acidity was not the cause of the altered pathogenicity profile demonstrated in these experiments. Chicken intestinal flora was present in the crude mucus and previously has been shown to reduce or delay chick colonization *in vivo* (Stern *et al.*, 2001). Although a role for chicken microflora in attenuating virulence in our experiments cannot be discounted, it is noteworthy that intestinal pathology does not usually occur when day-of-hatch chicks are experimentally infected (Beery *et al.*, 1988), suggesting that the acquisition of normal gut flora alone is unlikely to account for the absence of pathology in poultry.

The nature or composition of the avian mucus itself provides perhaps the most attractive candidate for the effect on the pathogenicity of *Campylobacter* observed in this study. Chicken mucins differ considerably from those of humans (Smirnov *et al.*, 2004; Verma *et al.*, 1994). Differences in mucus structure, folding, glycosylation, charge or associated molecules such as electrolytes could account for a 'trapping' effect of chicken compared with human mucus. Sputasol liquefaction failed to reverse the mucus-induced inhibition of bacterial cell association, suggesting the effect is not simply related to viscosity. However, previous work on *C. jejuni* (Szymanski *et al.*, 1995), and more recent experiments using *Pseudomonas* (Wolfgang *et al.*, 2004), provide strong support for the concept that mucus can have a direct effect on motility and virulence. Furthermore, it has been shown that the related gastrointestinal pathogen, *H. pylori*, binds specifically to TFF1, a human trefoil peptide found in gastric mucus (Clyne *et al.*, 2004). Further exploration of the composition and constituents of chicken mucus is warranted to explain how, in avian hosts, an intestinal mucosal environment has evolved that not only tolerates, but possibly even promotes the growth of *Campylobacter* while simultaneously defending the avian host from pathological effects.

In summary, using a primary cell model, we have shown that the lack of adherence of *C. jejuni* to chicken intestinal epithelium *in vivo* does not result from lack of cell surface receptors. Some extra cellular factor acting to interrupt attachment in colonized birds may explain the lack of cell association *in vivo*. We found chicken intestinal mucus markedly attenuated *C. jejuni* virulence compared with human mucus. Knowledge of the basis of the differential effect of chicken and human mucus on pathogenic behaviour in *Campylobacter* has implications for understanding the nature of commensalism in the gastrointestinal tract and may provide novel opportunities to interrupt the transmission of this zoonotic pathogen to humans.

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