BACTERIAL PATHOGENICITY

Characterisation of cytolethal distending toxin (CDT) mutants of Campylobacter jejuni

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In order to assess the contribution of cytolethal distending toxin (CDT) to the toxigenicity and pathogenicity of Campylobacter jejuni, the C. jejuni 81-176 and C. jejuni NCTC 11168 CDTs were inactivated by insertional mutation of the cdB toxin subunit. Cell-free sonicates from isogenic C. jejuni 81-176 cdB− strains were found to be greatly attenuated in HeLa cytotoxicity assays, whilst still retaining some toxigenicity. Sonicates from a C. jejuni NCTC 11168 cdB− strain produced no detectable cytotoxicity. When orally administered to adult severe combined immunodeficient (SCID) mice, C. jejuni cdB mutant strains were unaffected in enteric colonisation abilities but demonstrated impaired invasiveness into blood, spleen and liver tissues. These data suggest that CDT may be the principal toxin produced by this species and that some C. jejuni strains may generate additional toxicigenic factor(s) distinct from CDT.

Introduction

Campylobacters are recognised to be among the principal causal agents of bacterial gastro-enteritis throughout the world [1]. Campylobacters have been demonstrated to produce a multi-subunit toxin, designated cytolethal distending toxin (CDT) [2]. This toxin is closely related to an emerging family of bacterial protein toxins produced by Escherichia coli [3–5], Shigella dysenteriae, S. boydii [6], Haemophilus ducreyi [7] and a more recently identified form from the peridontal pathogen Actinobacillus actinomycetemcomitans [8, 9].

CDT has been shown to affect F-actin assembly and cell division in Chinese hamster ovary (CHO) cells [10] resulting in a characteristic elongation and swelling of susceptible cells over a 72–96 h period [11], distinct from the effects of other extracellular bacterial toxins. Partially purified CDT preparations have also been demonstrated to induce a profuse watery diarrhoea in suckling mice [12]. Recently, the mode of action of the E. coli CDT has been identified as affecting the cyclin regulatory unit, cdc2 protein kinase [13, 14]. CDT acts by preventing dephosphorylation of the cyclin B1/cdc2 protein kinase complex, which controls the entry of eukaryotic cells into mitosis [15]. This subsequently leads to a G2/M phase cell cycle block that results in swelling of affected cells and death 3–5 days after exposure.

An earlier report described an experimental infection model for campylobacters in adult immunodeficient mice [16]. Immunodeficient SCID-Beige mice infected with Campylobacter jejuni became rapidly and heavily colonised by campylobacters, thus providing a potential in-vivo model for assessment of the contribution of CDT to the pathophysiology of campylobacter infections.

Recently, a H. ducreyi cdC mutant has been characterised [17] and shown to be as virulent as wild-type bacteria in a rabbit model of experimental chancroid, despite producing no detectable CDT in tissue-culture assays. Given the paucity of knowledge and conflicting opinions concerning toxin production by campylobacters [18], the present study sought to investigate the contribution of CDT to the toxigenicity and pathogenicity of campylobacters, through the generation of toxin-deficient mutants. These mutants have been characterised for their ability to produce toxin active against HeLa and INT 407 cell lines and their ability to colonise and invade an adult SCID mouse model.
Materials and methods

Bacterial strains and plasmids

*C. jejuni* NCTC 11168 [19], *C. jejuni* NCTC 12744 [20] and *C. jejuni* 81-176 [21] have been described previously. *E. coli* DH5α and *E. coli* TOP10 were used as hosts in cloning experiments. Plasmids pCR2.1TO-PO (Invitrogen) and pMTL22 [22] were used as cloning vectors.

Media and growth conditions

Campylobacters were grown routinely on either Columbia blood agar (CBA) containing defibrinated horse blood 10%, cefoperazone-charcoal-desoxycholate-ampicillin (CCDA) agar, Mueller-Hinton (MH) agar or MH broth (Unipath) under micro-aerobic conditions (CO₂ 5%, O₂ 5%) in an ASSAB CO₂ incubator (Don Whitley Scientific). *E. coli* was cultured at 37°C on yeast tryptone agar or in broth. When necessary to maintain plasmid selection, ampicillin was added to growth media at a final concentration of 50 mg/L and chloramphenicol (Cm) was added at a final concentration of 20 mg/L. All *C. jejuni* strains utilised in experimental procedures were low passage isolates stored as frozen stocks at −70°C on Microbank cryobeads (Prolab Diagnostics). Loss of virulence factors following prolonged passage or storage of strains has been suggested as one reason behind the often contradictory reports by different groups concerning toxin production by campylobacters [18] and, accordingly, experimental procedures were designed to minimise the in-vitro passage of isolates. All wild-type and mutant *C. jejuni* strains used in toxicity assays were passaged on fewer than four occasions. Mutations were generated in wild-type bacteria following a maximum of two passages to minimise the potential down-regulation of virulence genes and adaptation to growth on laboratory media. Campylobacters were recovered by plating on to CBA agar. Single colonies were then transferred to fresh CBA plates and incubated micro-aerobically for a further 24 h before use.

DNA methods

All restriction endonucleases, T4 DNA ligase and alkaline phosphatase were purchased from Roche Diagnostics and used according to the manufacturer’s instructions. Plasmid DNA was isolated from *E. coli* by Qiaprep spin mini columns or Qiafilter maxi columns (Qiagen). All PCR cloning procedures with pCR2.1TO-PO were performed according to the manufacturer’s instructions (Invitrogen). Genomic DNA was prepared from campylobacters as described by Pitcher et al. [23]. Electrotransformations of campylobacters were done by the procedures described by Wassenaar et al. [20].

PCR

PCRs to amplify specific fragments from *C. jejuni* cdtABC genes were performed with primers based on *C. jejuni* 81-176 cdtABC sequence [2] as follows; primers P5 5’ CTTATCTAAAAGGGTACGC3’ and P9 5’ CATATCAAGGTTCATTATGC 3’ were used to amplify a promoterless *cdtABC* operon from campylobacters; primers P5 5’ GAAATCGTTGG CACCTTGGAATTGCAGGC 3’ and P6 5’ GAGT TCGTAAAATCCCCCTGATCATTCA 3’ were used to amplify a region internal to the *cdtB* gene from campylobacters. This DNA fragment was also used as a probe in later DNA–DNA hybridisations. All PCR reagents were supplied by Applied Biosystems. PCR reactions were carried out in 100-μl volumes and contained 0.125 μM (each) dATP, dCTP, dGTP and dTTP, 1X AmpliTaq PCR buffer, 100 pmol of each primer, 0.1 μg of template DNA and 2 units of AmpliTaq DNA polymerase. Reactions were cycled with an MJ Research Hot Bonnet thermal cycler as follows: 94°C for 8 min, followed by 30 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 2 min and a final extension step of 72°C for 8 min. A PCR-amplified chloramphenicol resistance cassette was used in mutagenesis experiments and was generated as described by Yao et al. [24].

DNA–DNA hybridisations were performed with ECL non-radioactive gene detection systems (Amersham) according to the manufacturer’s recommendations.

Cell cytotoxicity assays

HeLa cytotoxicity assays were done essentially as described by Pickett et al. [4], with slight modification. Briefly, campylobacters were grown micro-aerobically overnight on MH agar, containing chloramphenicol where appropriate. Cells were harvested, suspended in PBS and pelleted at 6000 g. Pellets were resuspended in 1 ml of PBS. The A₉₀₀ of the resuspended cells was then determined. Cell suspensions were sonicated (Soniprep 150, MSE) for two 30-s bursts with a 30-s interval. After sonication, samples were centrifuged (6000 g) and supernates were filtered (0.22 μm pore size). CDT assays were performed with HeLa and INT407 cells grown in Eagle’s minimal essential medium (EMEM) supplemented with fetal bovine serum 10%. Microtitration plates (96-well) were seeded with c. 2 × 10⁵ cells (100 μl/well) and incubated for 18 h before the assay was performed. Toxic titres were determined by performing a two-fold dilution series of cell sonicates in EMEM medium; 100 μl of each dilution were then applied to the HeLa and INT 407 cell microtitration plates. Titres were calculated by dividing the highest dilution to affect 50–75% of the cells in a well by the A₉₀₀ of the resuspended *C. jejuni* cells.
C.B 17-SCID-Beige mouse intragastric challenge

Wild-type and isogenic C. jejuni 81-176 cdB- mutants were administered intragastrically to groups of adult C.B-17-SCID-Beige mice at doses of 10⁷ cfu/mouse, according to the procedures described by Hodgson et al. [16]. Groups of five mice were then killed at 2, 6 and 24 h after challenge and blood, spleen, liver and faecal samples were removed and weighed. Control mice, uninfected with C. jejuni 81-176, were killed at equivalent time points. Viable counts were determined directly from blood samples plated on to CBA as described above. Liver and spleen samples were macerated in Ringer’s solution in a Griffiths tube, before plating on to CCDA agar and CBA for viable counts. Remaining blood and macerated liver and spleen samples were incubated for 48 h in Brucella broth, before plating on to CCDA agar for detection of low levels of C. jejuni. Faecal samples were homogenised by stomaching, before serial dilution and plating on CCDA agar to determine intestinal colonisation levels in experimental animals. Histological examination of SCID mouse large intestine samples was done as described previously [16].

Results

Generation of CDT+ mutants

PCR amplification of a promoterless cdABC operon was done with primers P8 and P9, based on the published nucleotide sequence from C. jejuni 81-176 [2]. A single fragment, corresponding in size to a promoterless cdABC operon, was generated from PCR reactions with C. jejuni 12744 template DNA. This fragment was subsequently cloned into pCR2.1TOPO, generating the vector pDP32, Sequence analysis of insert DNA from pDP32 indicated the successful cloning of a promoterless copy of the C. jejuni 12744 cdAB operon. In order to generate a vector suitable for mutagenesis of the C. jejuni cdABC operon, a PCR-amplified PpuII flanked chloramphenicol resistance cassette [24] was cloned into the unique HpaI site centrally located within the C. jejuni 12744 cdB gene. The resulting vector, pDP46, carried an insertionally inactivated cdB gene, but lacked sequences that would allow its extra-chromosomal maintenance in Campylobacter spp. Nucleotide sequencing results suggested that sufficient cross-strain homology was likely to exist for this vector to be utilised in mutagenesis experiments with a number of different C. jejuni strains. Restriction analysis indicated that the cm resistance cassette was inserted in the opposite orientation to the promoterless cdABC operon in pDP46 (data not shown).

Electroporation was used to transfer an insertionally inactivated cdB allele to wild-type C. jejuni strains by homologous recombination. Cm-resistant transformants were initially screened by PCR with primers P5 and P6, which flank the insertion site within the cdB gene. All C. jejuni 11168 and C. jejuni 81-176 clones screened generated a single 1.2-kb PCR product, c. 800 bp larger than wild-type bacteria, corresponding to the successful insertion of the Cm resistance cassette by double cross-over. However, all C. jejuni 12744 Cm-resistant clones generated a single PCR amplification product of identical size to that from wild-type bacteria, indicating that a single crossing-over event may have taken place, or that cdB sequences harbouring the Cm resistance cassette may have illegitimately recombined back into the chromosome.

DNA hybridisations

To confirm the findings of initial PCR screens of putative cdB mutants, two oligonucleotide probes were used to determine the presence of the Cm-resistance cassette within the cdB gene of C. jejuni 11168 and C. jejuni 81-176 and to confirm the presence or absence of plasmid sequences within the chromosome of Cm-resistant strains. The non-radioactively labelled cdB probe consisted of a PCR-amplified fragment internal to the C. jejuni 11168 cdB gene generated with the primer pair P5/P6. This hybridised to BglII/EcoRV-restricted DNA fragments, which appeared c. 800 bp larger from cdB mutants than from wild-type C. jejuni 11168 and C. jejuni 81-176 strains (Fig. 1), corresponding to the successful insertion of the Cm cassette within the cdABC operon. Both C. jejuni cdB mutants also demonstrated a decrease in fragment size hybridising to labelled cdB probe when digested with BscI, as a result of the presence of a BscI site within the Cm-resistance cassette. Labelled pCR2.1 backbone failed to generate any signal under low stringency hybridisation conditions (data not shown) in duplicate blots, indicating that allelic exchange had occurred by double cross-over.

HeLa cytotoxicity assays

It has been shown previously that cloned C. jejuni cdA, cdB and cdC genes are all required to produce active CDT in non-toxigenic E. coli K12 clones [2]. To assess the contribution of CDT to the overall cytotoxicity of C. jejuni, both mutant strains harbouring insertions within the cdB genes were assayed for cytotoxicity in HeLa cell assays. Serial dilutions of cell-free sonicates were applied to HeLa cell monolayers in 96-well microtitration plates. After incubation for 96 h, cytopathic effects (CPEs) consistent with the activity of CDT were visible with both the wild-type C. jejuni strains assayed. Toxin titres of 4096 and 512 were generated by C. jejuni 81-176 and C. jejuni 11168 strains, respectively. However, in isogenic C. jejuni strains harbouring insertions in the cdB gene, toxin titres were either greatly reduced or completely abolished, with the C. jejuni 81-176 cdB mutant generating a toxin titre of 8 and the C. jejuni 11168 cdB mutant generating no detectable cytotoxicity. This
absence of cytotoxicity in HeLa assays of the *C. jejuni* 11168 cdhB mutant leads to the speculation that CDT is the principal toxin produced by this strain under the growth conditions employed.

In contrast, *C. jejuni* 81-176 cdhB mutants still elicited cytopathic effects on the HeLa cells, albeit at greatly reduced titres. Typical morphology of HeLa cells was markedly different from CDT-induced CPE, indicating that another as yet undefined toxin may be active within these cells (Fig. 2). Cell-free sonicates imparted a distinctly elongated, spindle morphology on HeLa monolayers, but without the characteristic swelling of the cell associated with the action of CDT. Interestingly, this CPE is similar in nature to that generated following exposure of CHO cells to *E. coli* LT and may be associated with previous observations of enterotoxin-like activity generated by campylobacters [25, 26].

When identical comparative cytotoxicity assays were performed with INT407 cell monolayers, data generated from these assays corroborated those from the HeLa cell assays. However, INT407 cells consistently generated reduced toxin titres compared with HeLa cells (data not shown), typically of the order of 50–75%. This is not surprising, as not all CDT-sensitive cell lines appear to display equivalent morphological effects [27] and may reflect potential differences in toxin receptor binding sites between the two cell lines. As such, HeLa cells may constitute a more useful choice of cell line for such toxicity assays.

**Fig. 1.** Southern hybridisation of *C. jejuni* 11168 and *C. jejuni* 81-176 wild-type strains and cdhB mutants. Lanes 1–4, BglII/EcoRV-digested chromosomal DNA from: 1. *C. jejuni* 11168 wild-type; 2. *C. jejuni* 11168 cdhB mutant; 3. *C. jejuni* 81-176 wild-type; 4. *C. jejuni* 8-176 cdhB mutant. Lanes 5–8, BsrI-digested chromosomal DNA from: 5. *C. jejuni* 11168 wild-type; 6. *C. jejuni* 11168 cdhB mutant; 7. *C. jejuni* 81-176 wild-type; 8. *C. jejuni* 8-176 cdhB mutant.

**Fig. 2.** Photomicrographs of Giemsa-stained HeLa cell monolayers: (A) treated with filtered *C. jejuni* 81-176 cell sonicates; (B) untreated control; (C) treated with filtered *C. jejuni* 81-176 cdhB mutant cell sonicates. Bar, 10 μm.
SCID mouse challenge

Intragastric administration of 10^6 cfu of wild-type and isogenic C. jejuni 81-176 cdkB mutant strains to adult C.B-17-SCID-Beige mice was done to establish the contribution of CDT to campylobacter pathogenesis. These mice are homozygous for a recessive mutation that impairs early lymphocyte development and results in an absence of mature B- and T-cell lymphocytes [16, 28], subsequently rendering them more susceptible to enteric colonisation by campylobacters. Following intragastric inoculation of mice, blood obtained by cardiac puncture, liver and spleen samples were removed at 2, 6 and 24 h after challenge to assay for the presence of invasive campylobacters. Table 1 shows the first detected presence of C. jejuni 81-176 strains in mouse blood and tissues. At 2 h after challenge, wild-type bacteria were present in more spleen, liver and blood samples from experimental animals (8 of 15) than in samples from mice infected with isogenic cdkB mutants (4 of 15). These results suggest that C. jejuni 81-176 cdkB mutants invade SCID mouse tissues less readily than wild-type bacteria, most noticeably evidenced by the absence of cdkB mutants in directly plated spleen samples, and suggest a possible role for CDT in campylobacter infection.

Intestinal colonisation levels were monitored by direct plating of faecal samples and colony counts from mutant and wild-type C. jejuni 81-176 strains were indistinguishable (data not shown). As anticipated, during the comparatively short duration of the experiment, no SCID mice infected with wild-type or isogenic C. jejuni 81-176 cdkB mutants became ill, or displayed any obvious clinical symptoms relating to campylobacter infection. A previous study with CB-17-SCID-Beige mice [16] demonstrated diarrhoeal symptoms and histopathological lesions typical of human campylobacteriosis only over more extended time periods. Microscopic examination of SCID mouse intestines 7 days after infection also failed to reveal any histopathological changes in mice infected with wild-type or mutant C. jejuni 81-176.

Table 1. Summary of C. jejuni 81-176 wild-type and cdkB mutant isolation from CB-17-SCID-Beige mice

<table>
<thead>
<tr>
<th>Time after challenge (h)</th>
<th>Sample type</th>
<th>C. jejuni 81-176 wild-type (out of 5 tested)</th>
<th>C. jejuni 81-176 cdkB mutant (out of 5 tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Spleen</td>
<td>3 D</td>
<td>1 E</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>4 D</td>
<td>3 D</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>1 D</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Spleen</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>3 D + 1 E</td>
<td>3 D + 1 E</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>Spleen</td>
<td>1 D</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>0</td>
<td>1 D</td>
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<td></td>
<td>Blood</td>
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D, detected by direct plating; E, detected by enrichment only.

Discussion

This report indicates that CDT may be the principal toxin produced by both C. jejuni clinical isolates utilised in this study when cultured in vitro. Cell cytotoxicity assays indicated that C. jejuni 11168 cdkB mutants produced no other toxin active against HeLa cells or INT407 cells under the experimental conditions used, whereas C. jejuni 81-176 cdkB mutants appeared to retain a low level of toxic activity, apparently unrelated to the action of CDT. This weak residual toxicity on HeLa monolayers may account for an as yet uncharacterised toxic activity previously associated with campylobacters [18] and suggests that some, but not all, campylobacters may indeed produce more than one toxin. Previous studies, although unable to provide genetic data on toxic elements, have described various putative campylobacter toxins active on several different cell lines, ranging from Shiga-like toxin activity [29], to a number of different cytotoxins [30–32] enterotoxins [33, 34] and a hepatotoxin [35]. Strain variation may account for some of these differences in toxin production, which may become clearer as new evidence of the genetic heterogeneity of the campylobacters emerges. Guerry et al. [36] have highlighted strain variations in the iron uptake systems of certain C. jejuni strains, suggestive of the fact that alternative iron acquisition systems may exist in these strains as a response to different pathogenesis strategies or occupation of different ecological niches. Similarly, less conserved metabolic functions, such as toxin production, may also differ significantly between strains. It is also perhaps unfortunate that the C. jejuni 11168 strain, for which complete nucleotide sequence is now available [www.sanger.ac.uk/Projects/C._jejuni/], generated no additional toxigenicity following insertional inactivation of its cdIBC operon.

The widespread presence of genes encoding CDT amongst members of the Campylobacter genus was recently demonstrated by Eyigor et al. [37], indicating that CDT may be intimately involved in successful colonisation processes and in the ability of the
organism to cause disease in man. Inactivation of cdhB subunits in C. jejuni strains leads to a decrease in the ability of bacteria to invade C.B-17-SCID-Beige mouse tissues, further implying a potential role for this toxin in campylobacter pathogenesis. Given the plasticity of the campylobacter genome, the ubiquitous presence of the cdhABC operon amongst campylobacters is suggestive of the need for toxin production for pathogenesis in a suitable host. Coupled with the observation that C. jejuni, the principal human pathogen of the genus, also appears to elicit more CDT than other Campylobacter species [2, 14], this provides further evidence for involvement of CDT in campylobacter pathophysiology.

Although none of the C.B-17-SCID-Beige mice displayed overt signs of clinical illness in terms of diarrhoea or changes in gut histopathology during this study, the experimental period used was much shorter than that in previous studies [16]. Suckling mice challenged intragastrically with E. coli culture supernates expressing cloned S. dysenteriae cdhABC genes and partially purified toxin have previously developed CDT-induced watery diarrhoea [12]. It is unclear at present whether this difference in susceptibility to the toxin is related to the age and strain of mice, if toxin expression in the SCID mouse gut is reduced, or if it represents functional differences between the C. jejuni and S. dysenteriae CDTs.

Some correlation also appears to exist between the numbers of invading C. jejuni and the transcriptional activity of the cdh promoter in vitro. Reporter gene fusions to the cdh promoter demonstrate maximum activity within 2 h of inoculation of batch cultures, followed by a rapid decline in promoter activity (unpublished observations). Wild-type C. jejuni 81-176 invaded the tissues and blood of experimental animals more frequently and in greater numbers than the cdhA mutants 2 h after challenge, coinciding with the potential maximum transcriptional activity of the cdh promoter. At this time point, wild-type bacteria were detected by direct plating in three positive spleen samples, whereas CDT- mutants were only present following enrichment in the spleen of a single animal, indicating that inactivation of CDT may contribute towards reduced invasiveness. The fact that the mutant C. jejuni strain was still invasive reflects the multifactorial nature of invasion in this species [38].

These studies have demonstrated a potential relationship between the production of CDT and the invasive abilities of C. jejuni strains in a SCID mouse model. A wild-type strain consistently generated high toxin titres and was more successful in invading mouse tissues; in contrast, isogenic cdhB mutants failed to produce detectable toxin in the case of the C. jejuni 11168 mutant, or at greatly reduced titres in the case of the C. jejuni 81-176 mutant, indicating successful inactivation of CDT. More work will be necessary to unravel the complex nature of campylobacter pathogenesis, particularly in relation to clearly defining determinants of invasion and the clarification of the role of other toxic factors in this process.

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References