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Role of Cytolethal Distending Toxin in Altered Stool Form and Bowel Phenotypes in a Rat Model of Post-infectious Irritable Bowel Syndrome

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Background/Aims
Campylobacter jejuni infection is a leading cause of acute gastroenteritis, which is a trigger for post-infectious irritable bowel syndrome (PI-IBS). Cytolethal distending toxin (CDT) is expressed by enteric pathogens that cause PI-IBS. We used a rat model of PI-IBS to investigate the role of CDT in long-term altered stool form and bowel phenotypes.

Methods
Adult Sprague-Dawley rats were gavaged with wildtype C. jejuni (C+), a C. jejuni cdtB knockout (CDT-) or saline vehicle (controls). Four months after gavage, stool from 3 consecutive days was assessed for stool form and percent wet weight. Rectal tissue was analyzed for intraepithelial lymphocytes, and small intestinal tissue was stained with anti-c-kit for deep muscular plexus interstitial cells of Cajal (DMP-ICC).

Results
All 3 groups showed similar colonization and clearance parameters. Average 3-day stool dry weights were similar in all 3 groups, but day-to-day variability in stool form and stool dry weight were significantly different in the C+ group vs both controls (P < 0.01) and the CDT- group (P < 0.01), but were not different in the CDT- vs controls. Similarly, rectal lymphocytes were significantly higher after C. jejuni (C+) infection vs both controls (P < 0.01) and CDT-exposed rats (P < 0.05). The counts in the latter 2 groups were not significantly different. Finally, c-kit staining revealed that DMP-ICC were reduced only in rats exposed to wildtype C. jejuni.

Conclusions
In this rat model of PI-IBS, CDT appears to play a role in the development of chronic altered bowel patterns, mild chronic rectal inflammation and reduction in DMP-ICC.

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Key Words
Campylobacter infections; Cytolethal distending toxin; Inflammation; Models, animal
**Introduction**

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterized by chronic or recurrent abdominal discomfort associated with altered bowel habits, abdominal distention and bloating. Another characteristic feature of IBS is that symptoms alternate over time, with irregular bowel form and frequency. While the true pathophysiology of IBS is unknown, an increasingly recognized hypothesis is the development of IBS (post-infectious IBS [PI-IBS]).

While the mechanisms for the development of PI-IBS remain unknown, studies suggest that the rate of IBS development after acute gastroenteritis varies between 4% and 31%, with 2 main unknown studies suggesting that the rate of IBS development following an episode of acute gastroenteritis (post-infectious IBS) is an increasingly recognized hypothesis. While initial criticism suggested that this was a linear relationship with increased stool weight and increased rectal sensitivity. While initial criticism suggested that this was a lingering effect of the gastroenteritis, long-term studies have demonstrated persistence years later. Neal et al demonstrated that 57% of PI-IBS subjects continued to meet Rome criteria for IBS 6 years after the onset of PI-IBS, supporting the hypothesis that an episode of gastroenteritis could produce a persistent long-term effect.

Many bacterial pathogens can produce IBS. However, *Campylobacter jejuni* is the most common bacterial cause of diarrheal illness in industrialized nations. Moreover, acute *C. jejuni* gastroenteritis is frequently identified antecedent to PI-IBS. Cytotoxins distending toxin (CDT) is common to many human enteric bacterial pathogens. In *C. jejuni*, CDT is a tripartite complex of the *cdtA*, *cdtB* and *cdtC* gene products. *cdtB* has homology with DNaseI-like nucleases, and is considered the active subunit. Microinjection or transfection of this subunit alone into host cells is sufficient to induce the holotoxin's effects. The CdtA and CdtC subunits function in the delivery of cdtB into target cells. Once cdtB is translocated into the nucleus, it induces double-stranded DNA damage and causes arrest at the G1/S or G2/M interface of the cell cycle, depending on the cell type, ultimately leading to cell death. One in vitro study demonstrated that CDT caused irreversible cell cycle arrest of HeLa (after 24 hours) and Caco-2 cells (after 48 hours) at G2/M phase, finally leading to their death.

In a newly developed rat model of PI-IBS, we showed that rats inoculated with *C. jejuni* develop persistent altered stool form and increased rectal intraepithelial lymphocytes (IELs) months after complete clearance of the initial infection. Further characterization demonstrated a reduction in deep muscular plexus interstitial cells of Cajal (DMP-ICC) in the post-infectious phase. These findings are similar to findings in humans with PI-IBS. In an acute study, rats inoculated with a *C. jejuni* strain carrying an insertional deletion of *cdtB* demonstrated mucosal damage equivalent to wildtype *C. jejuni*. Thus, *cdtB* appears less important acutely. In this report, we examined the role of CDT in the development of long-term altered stool form, bowel patterns, rectal IEL elevation and DMP-ICC after clearance of *C. jejuni* infection.

**Materials and Methods**

**Campylobacter Infection of Sprague-Dawley Rats**

Adult Sprague-Dawley rats were obtained and fresh stool was cultured to establish the absence of *C. jejuni* on BBL™ *Campylobacter* Agar with *5* antibiotics and 10% sheep blood (Containing the antibiotics: Amphotericin B, Cephalothin, Trimethoprim, Vancomycin and Polymyxin B; BD Diagnostics, Franklin Lakes, NJ, USA) at baseline. Prior to infection, rats received a 1 mL oral gavage of 5% bicarbonate solution to transiently reduce the gastric acidity. One group of rats (n = 54) received a 1 mL gavage of 10⁸ CFU/mL *C. jejuni* 81-176 strain (C+). A second group of rats (n = 54) received a 1 mL gavage of 10⁸ CFU/mL of a mutated *C. jejuni* 81-176 that does not express functional *cdtB*, due to an insertion of the kanamycin gene (CDT−). A third group of normal Sprague-Dawley rats (n = 25) were gavaged with saline vehicle only (controls). The study was approved by the Cedars-Sinai Institutional Animal Care & Use Committee.

**Tracking Acute Colonization by Campylobacter jejuni**

After gavage, fresh stool was collected daily from infected animals. The stool specimens were cultured to establish the absence of *C. jejuni* on Campylobacter selective agar plates. The number of rats with at least 1 day defined successful intestinal colonization with detectable *C. jejuni* in stool. The number of days from inoculation to first detection of *C. jejuni* in the stool was then recorded to determine time to successful colonization. Stool cultures continued to be performed on a daily basis until 2 consecutive days with...
negative cultures was seen. The time from gavage to this point was marked as the clearance time.

**Determination of Post-infectious Phenotype**

Overall, rats were housed for 4 months. This included a 1 month post-gavage (infectious phase) and a 3-month post-infectious phase. At the end of this 4-month period, fresh stool was collected for 3 consecutive days from all rats and graded for appearance and consistency. A stool consistency score (modified Bristol Stool score) was used for grading stool. Normal stool was graded as 1; soft and poorly formed stool was graded as 2; and watery stool was graded as 3. The collected stool was weighed and desiccated. This was accomplished by measuring stool weight before and after oven drying (24 hours at 70°C in the presence of desiccant) and percent liquid contents were calculated. After the 3-day stool collection, the rats were euthanized by CO2 asphyxiation and pneumothorax. Laparotomy was performed and the rectum was dissected, fixed in 10% formalin, imbedded in paraffin, then cut and mounted for histological evaluation as previously described. A section of ileum was similarly resected at a location 5 cm proximal to the ileocecal valve for staining of DMP-ICC as described below.

**Rectal Intraepithelial Lymphocytes**

After rectal tissue was fixed in formalin and sectioned for slides, sections were stained using CD3 antibody (Dako, Carpinteria, CA, USA) to visualize the presence of IEL as previously seen in humans with PI-IBS. At this point, all slides were randomized and coded. The slides were then examined by readers blinded to the group allocation. The reader was asked to count the number of IELs as a function of the number of epithelial cells.

**C-kit Staining of Deep Muscular Plexus Interstitial Cells of Cajal**

Sections of ileum from each paraffin block were stained immunohistochemically using polyclonal rabbit anti-human CD117, c-kit (DakoCytomation, Carpinteria, CA, USA). The positive control used to test the quality of the stain was a c-kit positive gastrointestinal stromal tumor. The numbers of DMP-ICC in the entire mounted segment of ileum were counted.

**Statistical Methods**

During the infectious period, the colonization and clearance time were compared between the C+ and CDT- groups using the Mann-Whitney U test. At the end of 4 months (in the post-infectious phase), the average stool form was graded over 3 days and wet weight among C+, CDT- and control rats were calculated using one-way ANOVA. Since IBS in humans is characterized by variability in stool form, we also compared the standard deviations of the stool form and wet weights between groups. The 3-day standard deviation represented the variability of the measurement. The number of rectal IEL’s was reported as a ratio to the number of epithelial cells. This was also compared between the 3 groups by one-way ANOVA. Since the numbers of DMP-ICC were not normally distributed, a Mann-Whitney U test was used to compare counts between groups.

**Results**

**Campylobacter Colonization Phase**

Prior to inoculation with *C. jejuni* 81-176, *C. jejuni* was not detected in the stool of any of the rats at baseline. Post-gavage, 9 rats infected with *C. jejuni* (C+ group) died within the first 3 days after gavage (8 due to severe gavage trauma and 1 due to a liver tumor). The remaining rats showed no evidence of gavage-induced trauma. Thus, a total of 124 rats (45 rats in C+ group, 54 rats in CDT- group and 25 control rats) were included for final data analysis. The number of rats with at least 1 day of detectable stool colonization by *C. jejuni* after inoculation was equal in the C+ and CDT- groups (Fig. 1A). In the C+ group, 38 out of 45 rats (84%) were colonized with *C. jejuni* and in the CDT- group, 47 out of 54 rats (87%) were colonized for at least 1 day after gavage.

Stool cultures were then followed over time. Figure 1B details and compares the colonization patterns of the 2 groups post-inoculation. The average time to first detectable *C. jejuni* in stool after gavage (colonization time) was greater in the C+ group (6.7 ± 4.5 days) than in the CDT- group (4.2 ± 2.6 days) (P < 0.05). However, when the period of time from the day of inoculation to the mean day of complete clearance of *C. jejuni* from the stool was compared between the 2 groups, it was found to be shorter in CDT- group (10.7 ± 4.8 days) than in the C+ group (12.6 ± 5.9 days) (P < 0.05). The longest period of colonization, 29 days, was observed in a rat in the C+ group.

**Post-infectious Phase**

Four months after inoculation with *C. jejuni*, the 3-day average percent wet weight of stool among the groups was not sig-
CDT Contributes to IBS Phenotypes in a Rat Model

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Figure 1. (A) Campylobacter colonization in rats gavaged with wildtype Campylobacter jejuni (C+) or a C. jejuni cytolethal distending toxin B (cdtB) knockout (CDT-). No significant difference was noted between C+ and CDT- groups. (B) Campylobacter colonization and clearance times in C+ and CDT- groups.

Figure 2. (A) Comparison of 3-day average stool wet weight in uninfected controls, wildtype Campylobacter jejuni (C+) and C. jejuni cytolethal distending toxin B (cdtB) knockout (CDT-) infected 4 months after gavage. No significant difference was seen between the groups. (B) Variability in stool wet weight over 3 days between groups. There was no statistical difference between control and CDT- rats.

When examining the average stool consistency (as measured based on a modified Bristol stool score) significant differences were observed between groups. From the data presented in Figure 3, the C+ group had a higher average stool consistency (score 1.51 ± 0.06) and thus less formed stool in comparison with the rats in the CDT- group (1.23 ± 0.03) (P < 0.0001) and controls (1.15 ± 0.05) (P < 0.0001), where 1.0 was normal. There was no statistical difference between the controls and CDT- rats.

In addition to average stool consistency, variability of stool
consistency over 3 days was also compared. In this analysis, C+ rats displayed the greatest daily variability of stool consistency (0.51 ± 0.06) compared to the variability of CDT- rats (0.30 ± 0.05) (P < 0.01) and controls (0.28 ± 0.08) (P < 0.05) (Fig. 4). The CDT- rats appeared unaffected, as their stool consistency was not statistically different from the control group.

Rectal Intraepithelial Lymphocytes

Differences were also seen in the number of rectal IEL in the 3 groups. The number of rectal IELs was highest in C+ rats (0.97 ± 0.15/100 epithelial cells) compared to controls (0.48 ± 0.11 per 100 epithelial cells, Table) (P < 0.01) and CDT- rats (0.59 ± 0.09/100 epithelial cells, Table) (P < 0.05) (Fig. 5). As with previous results, the CDT- rats were not statistically different from controls.

Deep Muscular Plexus Interstitial Cells of Cajal

The DMP-ICC staining from the 3 groups was compared. In each case, the numbers of DMP-ICC in the entire mounted segment of ileum were counted. Months after clearance of infection, C+ rats had a noticeable and consistent reduction in DMP-ICC (0.152 ± 0.121) (Fig. 6C) when compared to controls (0.218 ± 0.159, Table) (P = 0.024, Fig. 6A). However in CDT- rats, DMP-ICC numbers were not only preserved but greater in number than in controls (0.892 ± 0.363, Table) (P < 0.001 compared both to controls and to C+, Fig. 6B).

Discussion

The results of this study demonstrate that rats inoculated with wildtype C. jejuni 81-176 (C+ rats) exhibit altered variability of stool form and wet weight 4 months after infection. In contrast, rats inoculated with a mutant C. jejuni strain that does not express cdtB (CDT- rats) exhibited bowel patterns that were closer to normal, as determined from uninfected control rats. In addition, rats exposed to wildtype C. jejuni demonstrated elevated rectal IELs and reduction in DMP-ICC, which was not seen in

| Table | Quantification of Rectal Intraepithelial Lymphocytes and Deep Muscular Plexus Interstitial Cells of Cajal in Rats Gavaged With Wildtype Campylobacter jejuni, a C. jejuni Cytolethal Distending Toxin B Knockout or Saline Vehicle |
|-------|-----------------------------------------------------------------------------------------------------|
|       | Controls                                                                                           | CDT-       | C+         | P-value             |
| No. of IELs (mean ± SD) | 0.480 ± 0.110                                                                                     | 0.590 ± 0.090 | 0.970 ± 0.150 | < 0.05<sup>b</sup> < 0.01<sup>c</sup> |
| No. of DMP-ICC Controls (mean ± SD) | 0.218 ± 0.139                                                                                     | 0.892 ± 0.363 | 0.152 ± 0.121 | < 0.001<sup>d</sup> 0.024<sup>c</sup> |

<sup>a</sup>Values given denote number of intraepithelial lymphocytes per 100 epithelial cells, <sup>b</sup>P-values are the difference between the control and CDT- groups, <sup>c</sup>P-values are the difference between the control and C+ groups.

CDT-, Campylobacter jejuni cytolethal distending toxin B knockout; C+, wildtype C. jejuni; IELs, intraepithelial lymphocytes; DMP-ICC, deep muscular plexus interstitial cells of Cajal.
CDT Contributes to IBS Phenotypes in a Rat Model

Figure 5. Immunohistochemical staining of rectal tissue from control (A), *Campylobacter jejuni* cytolethal distending toxin B knockout (CDT-, B) and wildtype *C. jejuni* (C+, C) rats with antibodies to CD3, to evaluate rectal intraepithelial lymphocytes. (D) Comparison of average intraepithelial rectal lymphocytes (per 100 epithelial cells) in uninfected controls, C+ and CDT- infected rats. There was no statistical difference between control and CDT- rats. IEL, intraepithelial lymphocytes; EC, epithelial cell.

Figure 6. Immunohistochemical staining of ileal tissue from control (A), *Campylobacter jejuni* cytolethal distending toxin B knockout (CDT-, B) and wildtype *C. jejuni* (C+, C) rats with CD117 to evaluate deep muscular plexus interstitial cells of Cajal (×20 magnification).

rats inoculated with the CDT- *C. jejuni*. In this PI-IBS rat model, 3 months after complete clearance of *C. jejuni* infection, CDT appears to be a determinant of the development of altered stool form, irregular bowel pattern and inflammation, but does not appear to affect the establishment of initial colonization during acute infection.
Development of IBS has been demonstrated in patients infected with numerous bacterial pathogens including Shigella, Escherichia coli and Salmonella. However, development of PI-IBS has been most thoroughly studied in patients who were infected with *C. jejuni*. The strongest risk factor for the development of PI-IBS appears to be prolonged duration of gastroenteritis (relative risk = 11.5). Human subjects with PI-IBS display the characteristic features of IBS, including altered stool form. Another physiologic hallmark of PI-IBS is increased rectal IEL counts. Spiller et al have demonstrated that 1 year after Campylobacter infection, the number of enteroendocrine cells and IELs (per 100 epithelial cells) in the rectum of PI-IBS patients remained significantly elevated compared to a control group (P < 0.001).

While animal models of PI-IBS are limited, there is a well-characterized model in a mouse previously infected with the parasitic pathogen Trichinella spiralis. In this model the focus has been on post-infectious gut neuromuscular dysfunction and visceral hyperalgesia. Although a human outbreak of *Trichinella britovi* from Turkey has demonstrated an overall 13.9% incidence of IBS following infection, this pathogen is not common in western countries.

In a recent study, we demonstrated that rats exposed to *C. jejuni* had greater altered stool form. Additionally, these rats demonstrated increased IEL counts in the rectum and left colon, but not in cecum or small bowel. This new rat model reproduces IBS phenotypes, including altered stool form and increased rectal lymphocytes.

Various lines of evidence suggest that during *C. jejuni* infection, CDT is a key virulent factor. Using a severe combined immunodeficient mouse model, Purdy et al demonstrated greater tissue (blood and liver) invasion and virulence by wild-type *C. jejuni* expressing CDT compared to *C. jejuni* CDT mutants, although the intestinal colonization levels remained equal in both 2 groups. This observation was further corroborated with a limited enteric flora mouse infection model. Fox et al demonstrated that *C. jejuni* CDT+ mutants failed to colonize the gastrointestinal tract of C57BL/129 mice (immune competent) in contrast to wildtype *C. jejuni* (0% vs 50%). In addition, using nuclear factor κB-deficient mice (3X), they also reported that, despite persistent and equal gastrointestinal colonization rates (100%) 3X mice infected with wildtype *C. jejuni* exhibited severe gastrointestinal inflammatory changes, compared to modest gastrointestinal inflammatory changes in mice infected with a cdtB mutant. These results imply that CDT may contribute to persistent gastrointestinal colonization, by playing a role in the ability of *C. jejuni* to escape immune surveillance in immunocompetent C57BL/129 mice (but not in 3X mice). One proposed mechanism for this phenomenon is its ability to cause cell cycle arrest of lymphocytes at G2/M phase, similar to its effect in epithelial cells. Also, less severe inflammation in 3X mice suggests that inflammation of the gastrointestinal tract is a feature of CDT activity. More recently, Jain et al demonstrated that suckling mice (which lack a fully mature immune system) inoculated with CDT+ *C. jejuni* developed pan-mural inflammation with mucosal denudation and necrosis affecting the jejunum, ileum and colon in all mice, compared to mice infected with cdtB- *C. jejuni*, which showed mild inflammatory changes only in the descending colon. These findings are consistent with in vitro studies reporting that CDT induces IL-8 from intestinal cell line INT407.

Our current study supports a longer colonization time among rats infected with wildtype *C. jejuni* vs CDT- *C. jejuni*. However, in a recently published study of acute histological changes in rats exposed to wildtype and CDT- *C. jejuni*, there very few histological differences during the acute infection during first 4 weeks after gavage. Both groups had significant disturbances including epithelial cell ballooning, villous blunting and villous tip disruption in the absence of cellular inflammation; these changes resolved by 3 months post-infection. Based on these findings, the role of CDT may involve mechanisms that are not related to the events surrounding the initial acute infection.

Various mechanisms of CDT action have been reported. For example, CDT may cause growth arrest of villous epithelial cells, thereby decreasing the barrier function of the intestinal epithelium and producing loss of nutrient absorption. It is also possible that cell cycle arrest of the intestinal epithelial cells would increase the contact time between the host and the pathogen. Whether CDT-deficient strains of *C. jejuni* produce less severe diarrheal disease and other chronic sequelae in human infections remains to be assessed directly. Many studies have reported the existence of higher frequency of cdtB+ *C. jejuni* compared to cdtB- strains, signifying that cdtB is present in majority of *C. jejuni* strains. In a recently concluded study, Jain et al demonstrated that cdtB+ *C. jejuni* strains adhere to and invade the HeLa cells, more effectively than the cdtB- strains. *C. jejuni* and other CDT-producing enteric pathogens may have effects on intestinal epithelial cells; specifically cells in the intestinal crypts resulting in rapid G2 phase cell cycle arrest of proliferating cells leading to their growth arrest. Thus, CDT may have profound effects on crypt cell maturation into functional villous epithelial
cells. This effect may also be responsible for a temporary epithelial injury and loss of absorptive functions, as suggested by a study reporting increased loss of tissue culture cells without recovery of the affected cells at the end of 72 hours when treated with C. jejuni CDT.44

The mechanism linking these in vitro toxic effects to prolonged bowel disturbance is uncertain. However, increases in inflammatory and enteroendocrine cells, observed in colonic mucosa of PI-IBS patients, could result in altered bowel patterns and changes in neuromuscular function.45 One interesting study showed that Campylobacter enteritis leads to loss of gut mucosal neural staining.44 Enteric neuronal damage and subsequent bowel dysfunction may result from immune mediated mechanisms that disturb the enteric nervous system. Our results support this possibility. In our study, CDT-C. jejuni did not result in loss of DMP-ICC, which suggests that CDT may have a role in degeneration of neuronal function in PI-IBS. Another notable example of neurotoxicity is Campylobacter-induced post-infective polyneuritis, known as Guillain-Barré syndrome, in which CDT has a neurotoxic effect. This intriguing possibility is supported by our recently published finding that wildtype CDT produces a reduction in DMP-ICC in this rat model.24 In contrast, CDT- rats showed DMP-ICC staining that was not reduced but increased compared to controls. This increase in ICC could be a response to infection in the absence of CDT; lack of growth arrest of ICCs or lack of cross-reacting immune response to ICCs may underlie this finding, but more work is needed to determine whether these or other mechanisms explain this observation. Future studies need to evaluate the effect of CDT on the gut neuromuscular apparatus.

In conclusion, using wild type and CDT- strains of C. jejuni in a post-infectious IBS rat model, we demonstrate that Campylobacter CDT is an important factor in the development of altered stool form, chronic altered bowel pattern, rectal inflammation and reduced DMP-ICC, since the absence of this toxin resulted in post-infectious phenotypes similar to those in uninfected control rats.

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