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THE ROLE OF CYTOLETHAL DISTENDING TOXIN IN HELICOBACTER HEPATICUS INDUCED MURINE GASTROINTESTINAL DISEASE

presented by

Jason Samuel Pratt

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of the requirements for the

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Major Professor's Signature

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THE ROLE OF CYTOLETHAL DISTENDING TOXIN IN HELICOBACTER HEPATICUS INDUCED MURINE GASTROINTESTINAL DISEASE

By

Jason Samuel Pratt

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ABSTRACT

THE ROLE OF CYTOLETHAL DISTENDING TOXIN IN HELICOBACTER HEPATICUS INDUCED MURINE GASTROINTESTINAL DISEASE

By

Jason Samuel Pratt

The gram-negative bacterium, *Helicobacter hepaticus*, can trigger inflammatory bowel disease (IBD) and hepatitis in mice. *H. hepaticus* expresses a member of the cytolethal distending toxin (CDT) family of bacterial cytotoxins. CDT is a DNA damaging toxin that causes cell cycle arrest in the G2/M phase, cellular enlargement and eventually cell death in cultured mammalian cells. Persistent infection with *Helicobacter hepaticus* can lead to chronic inflammation and neoplasia in mice. We explored the role of CDT in the etiopathogenesis of *H. hepaticus* induced murine gastrointestinal disease using isogenic CDT mutants. CDT was not required to initiate inflammatory bowel disease (IBD), but led to an increase in disease severity. In long-term cancer studies, CDT producing strains of *H. hepaticus* were able to cause an increased incidence of colon tumorigenesis in an IBD model, and hepatic tumorigenesis in a hepatitis model when compared to control mice and those challenged with a CDT deficient strain. CDT production by *H. hepaticus* is required for long-term persistence in mice. Clearance of the CDT deficient strain from mice led to a protective immunity upon rechallenge with both wildtype and the CDT deficient mutant of *H. hepaticus*. These results suggest that CDT plays two roles in the pathogenesis of *H. hepaticus* infection; a proinflammatory role as well as an immunosuppressive role that allows persistence of the organism.

This work is dedicated to my family and friends

ACKNOWELDGEMENTS

I would like to acknowledge my lab for all of their help in my tenure as a graduate student. I would also like to give special thanks the USDA National Needs fellowship for funding my research, my committee and Dr. Young for their guidance, and the support staff that aided my work including the Histology Lab and NFSTC Staff.

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Introduction

Helicobacter species are responsible for a wide range of acute and chronic human and veterinary infections (25). A subset of this genus, the enterohepatic *Helicobacter* species (EHS) encompasses a number of emerging pathogens that colonize the intestinal tract and/or livers of humans, a variety of other mammals and birds (10, 22, 25). They are the etiologic agents of gastroenteritis, hepatitis, and bacteremia. All *Helicobacters* are gram-negative, microaerobic, motile, curved to spiral rod shaped bacteria (11). The EHS have shown an ability to persist long–term in a chronic infection. These organisms have been associated with chronic inflammation and neoplasia. A parallel can be drawn with the EHS and the relationship between chronic gastritis and gastric cancer associated with *H. pylori* infection in humans (1).

Helicobacter hepaticus is a murine pathogen that naturally infects the distal gastrointestinal tract of mice, with high levels of colonization in the cecum. It was originally discovered in control A/JCr mice used as a part of a long-term cancer study. These controls developed much higher rates of liver cancer and hepatitis than expected. It was later discovered that *H. hepaticus* was the causative agent for the development of the chronic hepatitis and hepatocellular cancer seen in these A/JCr mice (11, 27). *H. hepaticus* has since been associated with the development of inflammatory bowel disease (IBD) in immune altered mice, and has been used as a model system for studying human IBD (3, 5, 14). Long-term infection with *H. hepaticus* in these immune-altered animals has been associated with the development of gastrointestinal neoplasias (7, 9).

In most strains of mice, *H. hepaticus* infection causes subclinical hepatic disease and enteritis. Mice with an altered immune function develop a severe and progressive typhlitis/colitis associated with *H. hepaticus* infection. This may lead to rectal prolapse, adenocarcinoma, weight loss and death (2, 3, 5, 8, 19). As a result, this animal model has been proposed as a model for studying human Crohn's disease.

H. hepaticus and a number of other EHS have been shown to produce a cytotoxin that is a member of the cytolethal distending toxin (CDT) family (4, 30, 32). Cytolethal distending toxin is a virulence factor produced by a host of pathogenic bacteria, including *Campylobacter jejuni,* and other *Campylobacters, Haemophilus ducreyi, Actinobacillus actinomycetemcomitans, Helicobacter hepaticus* and certain strains of *Escherichia coli* (reviewed in (6, 17, 21)). CDT causes cell cycle arrest in the G2/M phase, resulting in cellular enlargement and eventually cell death in effected mammalian cells. CDT is an AB toxin. The functional CDT holotoxin is composed of the product of three genes, *cdtA, cdtB*, and *cdtC* (15, 20, 24) with CdtB constituting the active (A) subunit, and CdtA and CdtC combining to makeup the (B) subunit. The *cdtB* gene product is homologous to type I deoxyribonucleases, while CdtA and CdtC share no homology with other known proteins. It has been hypothesized that CdtA and CdtC are involved in delivering CdtB to the cells (16). This working hypothesis states that CdtB forms a tripartite complex with CdtA and CdtC which allows the holotoxin to bind to the cell and facilitates entry of the toxin (20).

Although the exact role of CdtC is unknown, it is suggested that this subunit is required for the assembly of active holotoxin or that it may work in coordination with CdtA to bind to the outer membrane (13). The crystal structure of CDT has now been

solved, and the N terminus of CdtC appears to create a steric block of the active site of CdtB. This inhibition of the CdtB DNase activity may be a self-regulatory mechanism for CDT. It could prevent premature DNase activity, that could potentially damage the bacterial cell producing the toxin (20). CdtA possess a region encoding for a signal peptidase II recognition site that is characteristic of interaction with lipoproteins. This provides evidence for an involvement in anchoring the holotoxin on the bacterial outer membrane.

CdtB is the most highly conserved subunit of the protein, showing little variation in its sequence across a number of bacterial species. Studies have shown that transient expression of *cdtB* in eukaryotic cells is sufficient to replicate the dramatic chromosomal aberrations and cell cycle arrest that is observed with extracellular treatment with the holotoxin. Also, microinjection of CdtB into the nucleus of a cell is sufficient to induce DNA damage and cell cycle arrest (18).

CdtB possess a nuclear localization domain, in the N terminus of the protein, that allows this subunit to target the nucleus where it can inflict damage on the DNA (16). It has been proposed that this DNase activity is responsible for the cell cycle arrest that is a key feature of CDT-mediated cytotopathic effect in vitro. The role of CDT in the in vivo pathogenesis of organisms that elaborate this toxin has been investigated. Initial studies using isogenic CDT mutants of *Haemophilus ducreyi* (26, 29) and *C. jejuni* (23) reported minimal attenuation of virulence. A more recent report demonstrated that wild-type *C. jejuni*, but not an isogenic mutant lacking CDT, triggered gastroenteritis in NF-κB-deficient mice (12).

The purpose of these studies is to determine the role of cytolethal distending toxin in murine gastrointestinal disease using isogenic CDT mutants of *H. hepaticus* in a model of 1) Crohn's and colitis that results from a TH1 mediated immune response in C57BL6 IL-10 knockout mice and 2) hepatitis and hepatolcellular cancer in A/J mice. By studying this murine model of inflammatory bowel disease, I hope to draw a correlation between the role of the bacterial virulence factors and the triggers of human gastrointestinal disease.

Materials and Methods

Mutant Construction

The cloned CDT gene cluster, as well as the surrounding region of the chromosome, were mutagenized via transposon mutagenesis in vitro, and cloned in *E. coli.* The transposon contained the gene chloramphenicol acetyl-transferase (*cat*), which confers chloramphenicol resistance. The sites of transposon insertion were then determined by restriction mapping and DNA sequence analysis. The isogenic mutants were tested for CDT activity in vitro. pVBY9::Tn16, in which the transposon is inserted outside of the CDT coding sequence was introduced into 3B1 (wildtype6 *H. hepaticus*) by high voltage electroporation to produce the CDT positive isogenic mutant 3B1::Tn16. pVBY9::Tn20, which contains the transposon inserted into the *cdtA* gene , was introduced into 3B1 to produce 3B1::Tn20, a CDT negative isogenic mutant (See Figure 1).

CDT assays

H. hepaticus was harvested from agar plates with a cell scraper and was resuspended in Hanks' balanced salt solution. Total bacterial proteins were solubilized with a nonionic detergent (bacterial protein extraction reagent [B-PER] according to the recommendations of the manufacturer. Bacteria were pelleted by centrifugation at 2,040 x *g* for 10 min, resuspended in 300 µl of B-PER, and vortexed. Insoluble material was removed by centrifugation at 13,800 x *g* for 5 min, and soluble proteins contained in the supernatant were collected and stored at –80°C. *H. hepaticus* growth from three 100-mm-diameter plates was harvested into 1 ml of phosphate-buffered saline and disrupted

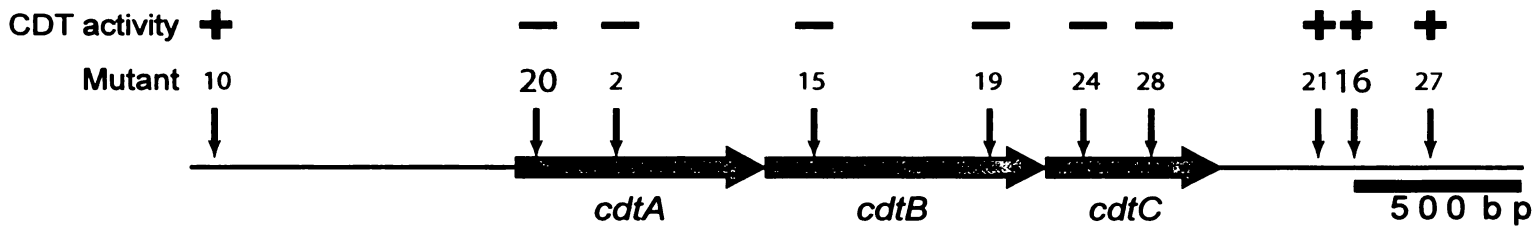

Figure 1. Map of the transposon insertions into the CDT gene cluster. A transposon insert downstream of the gene cluster at location 16, (3B1::Tnl 6), retained functional CDT cytotoxic activity. Transposon insertion into location 20, (3B1::Tn20), disrupted the gene cluster resulting in a loss of CDT cytotoxic activity.

by six 30-s pulses on ice. Debris was removed by centrifugation, and the cleared sonicates were filtered through a 0.2-μm-pore-size filter before being stored at –80°C.

HeLa cells were seeded onto 13-mm-diameter circular glass coverslips in 24-well tissue culture plates or in 25-cm^2 tissue culture flasks treated with B-PER extracts, sonicates from wild-type *H. hepaticus*, or the isogenic CDT mutants at a density of 2×10^3 per well. Twenty microliters of bacterial sonicate was added to each well, and the plates were incubated in 5% CO_2 at 37°C. CDT activity was determined by direct microscopic examination of stained monolayers or cell cycle analysis by flow cytometry (See Figure 2).

Bacterial Strains and Cell Lines

The wild type *H. hepaticus* strain 3B1 (the type strain, ATCC 51488) was obtained from the American Type Culture Collection (Manassas, VA). The isogenic mutants 3B1::Tn16 and 3B1::Tn20 were generated by transposon mutagenesis (42). 3B1::Tn20 has a transposon inserted near the start of *cdtA* and no longer produces cytolethal distending toxin. 3B1::Tn16 has a transposon inserted downstream of *cdtC* in a putative intragenic region and still produces functional cytolethal distending toxin (42). Wild type *H. hepaticus* and isogenic mutant strains were grown at 37°C for 3 to 4 days in a microaerobic environment, which was maintained in vented GasPak jars without catalyst after evacuation to –20 mm Hg and equilibration with a gas mixture consisting of 80% N2, 10% CO2, and 10% H2. *H. hepaticus* was grown on tryptic soy agar (TSA) supplemented with 5% sheep blood and with 20 μg/ml chloramphenicol (all from Sigma, St. Louis, MO) for the chloramphenicol-resistant transposon mutants.

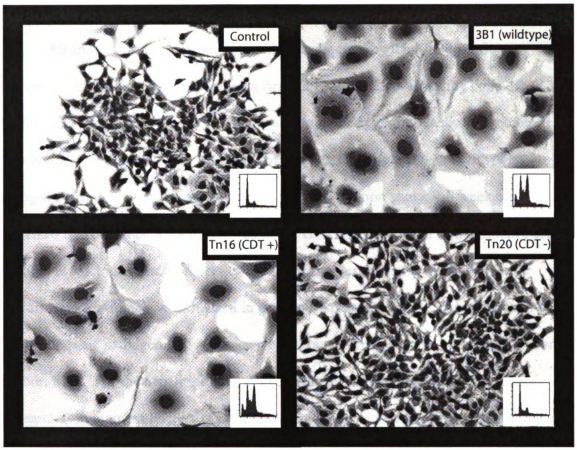

Figure 2. Cytopathic effect in HeLa cells72 hours after treatment with bacterial extracts. Compared to control cells, cells treated with detergent extracts from wild type *H. hepaticus* strain 3B1 exhibit cytoplasmic and nuclear enlargement along with nuclear abnormalities. Cell cycle analysis demonstrated G_2/M arrest (Data not shown). The isogenic mutant 3B1::Tn16 (with the insertion site downstream of the CDT gene cluster) retained the ability to produce cytopathic effect and cell cycle arrest, whereas 3B1::Tn20 (with the insertion site at the beginning of the *cdtA* gene) lost both of these abilities.

Animals

All animal protocols were reviewed and approved by the Michigan State University All University Committee on Animal Use and Care. Breeding pairs of *Helicobacter*-free C57BL/6 IL-10-/- mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed with autoclaved food, bedding and water with cage changes performed in a laminar flow hood. For the infection study, 4-6 week old C57BL/6 IL-10-/- mice were transferred to the University Research Containment Facility (URCF) at Michigan State University and housed under the same conditions as the breeding pairs. Animals were housed in groups of up to 5 animals per microisolator cage.

Specific pathogen free A/J mice were obtained from Jackson Laboratories (Bar Harbor, ME). For the hepatitis study, 8 week old A/J were housed at the University Research Containment Facility (URCF) at Michigan State University and housed under the same conditions as described above.

Murine Infection with *H. hepaticus*

H. hepaticus was harvested after 48 h of growth on agar plates and resuspended in a small volume of tryptic soy broth. The optical density (OD) at 600 nm of the inoculum was measured and ten-fold serial dilutions of the inoculum plated to quantify the CFU used for infection. Mice were inoculated with a single dose of a suspension of bacteria with an OD of 1.0 at 600nm (~1 x 108 CFU) in a volume of 0.2-0.3 mL. Bacteria were introduced directly into the stomach with a 24-gauge ball-tipped gavage needle. Control mice were inoculated with sterile tryptic soy broth.

Detection of *H. hepaticus* in Mouse Feces and Tissues

Fresh fecal pellets were collected from each mouse. Culture for *H. hepaticus* was accomplished by homogenizing feces in 0.5 mL of PBS and plating 50 μL on TSA supplemented with 5% sheep blood, 20 μg/mL cefoperazone, 10 μg/mL vancomycin and 2 μg/mL amphotericin B (TSA-CVA). DNA was isolated from fecal pellets as described previously (36). DNA was isolated from gastrointestinal tissue collected at the time of necropsy using a commercial kit (Qiagen Tissue Kit, Valencia, CA).

Direct, single-stage PCR amplification to detect *H. hepaticus* was performed using the primers 5' GCA TTT GAA ACT GTT ACT CTG 3' (B38) and 5' CTG TTT TCA AGC TCC CC 3' (B39) that produce a 417-bp amplicon (31). PCR was performed using 5 μL of template at approximately 100 ng/μl of DNA extracted from fecal or tissue samples. Each 25 μL PCR reaction contained 20 pmol of each primer, 200 μM of each dNTP, and 1.5 U of *Taq* DNA polymerase in a final concentration of 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl2 (Ready To Go PCR beads; Amersham Pharmacia Biotech, Piscataway, NJ). Cycling conditions included 30 cycles of 30 seconds at 94°C, 45 seconds at 54°C and 45 seconds at 72°C. PCR products were visualized by agarose gel electrophoresis. To provide a measure of relative levels of colonization with *H. hepaticus*, a nested PCR system was developed (See Figure 3). The first stage PCR employs primers 5' GCT ATG ACG GGT ATC C 3' (C97) and 5' ACT TCA CCC CAG TCG CTG 3' (C05) that amplify the small-subunit rRNA gene from all known *Helicobacter* species (10), yielding an approximately 1200 bp amplicon. PCR was performed using Ready To Go PCR

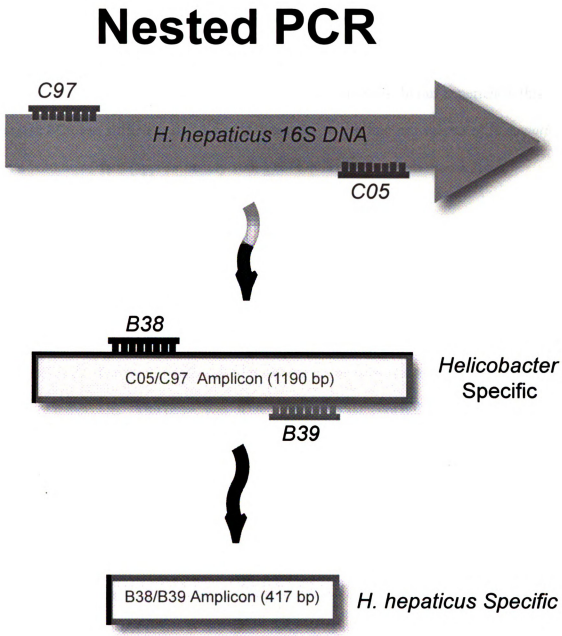

Figure 3. Nested PCR used to detect the presence of *H. hepaticus*. Total DNA was extracted from fecal pellets and cecal tissue. *Helicobacter* specific primers C05/C97 amplify a portion of the 16S gene. In order to increase sensitivity and specificity, the C05/C97 amplicon is used as a template for *H. hepaticus* specific primers B38/B39.

beads. Cycling conditions included 30 cycles of 30 seconds at 94°C, 60 seconds at 58°C and 90 seconds at 72°C. The nested stage was performed using primers B38 and B39 described above using 1 μL of the reaction mixture from the primary PCR as template. PCR products were visualized by agarose gel electrophoresis. In our experience, this nested PCR has sufficient sensitivity to detect 25-50 genome equivalents of *H. hepaticus* in a volume of 1μL (approximately 100ng) of DNA purified from mouse feces.

Mouse Necropsy and Histology (IBD)

Mice were euthanized by CO_2 asphyxiation at six weeks post infection. In order to remove the ileocecocolic junction, the peritoneal cavity was opened and the cecum externalized. The terminal ileum and proximal colon were then transected in order to remove the cecum. The tissue was placed in a petri dish containing phosphate buffered saline and its contents were expressed using the back of a razor blade. The tissue was then placed into a histological tissue cassette in neutral buffered formalin (10%). Once fixed, the tissue was processed and paraffin embedded. The tissue blocks were sectioned so that the lumen of the ileocecocolic could be viewed. Tissue sections were placed on glass slides and stained with hematoxylin and eosin.

Histologic section were encoded to prevent bias and scored for inflammation using the following system: 0 normal; 1, small multifocal lamina proprial and/or transepithelial leukocyte accumulations; 2, coalescing mucosal inflammation with or without early submucosal extension; 3, coalescing mucosal inflammation with a prominent multifocal submucosal extension with or without follicle formation; and 4, severe diffuse

inflammation of the mucosa, submucosa, and deeper layers (31).

Mouse Necropsy and Histology (Hepatitis)

Mice were maintained for 18 months to determine the effects of *H. hepaticus* on long-term infection of wildtype mice. One mouse per cage was euthanized by CO_2 asphyxiation at 9 months post infection, while the remainder were euthanized at 18 months. The peritoneal cavity was opened in order to expose and externalize the liver. The liver was then observed to determine the presence or absence of noticeable tumor formation. The liver was then removed with gall bladder attached and placed in neutral buffered formalin (10%). Upon fixation, the lobes were separated using a razor blade. The lobes were transected laterally and the sections placed in a histological tissue cassette. The tissue was processed and paraffin embedded. Tissue sections were placed on glass slides and stained with hematoxylin and eosin. In order to view *H. hepaticus* within the tissue, a spirochete specific Steiner stain was also employed. Tissue sections were blinded and scored by a veterinary pathologist.

Blood samples were also collected from the heart as follows: Upon opening the peritoneal cavity, the diaphragm was punctured. The rib cage was transected using scissors, exposing the heart. A 25-guage needle was inserted into the left ventricle of the heart in order to extract a sample. The average sample collected ranged between 200-500 µl. Samples were then spun at 10,000 x g for 10 min to separate the serum. Serum samples were removed and stored at -80 °C.

Preparation of *H. hepaticus* Antigens

H. hepaticus was cultured on TSA blood plates and suspended in PBS at O.D.600 1.5-3.0. The suspension was then frozen at -70°C, thawed, and pelleted at 5,000 RPM for 10 minutes. The pellet was resuspended in B-PER I reagent (Pierce Chemical Co., Rockford, IL, USA) then centrifuged at 13,000 RPM for 5 minutes. The protein concentration of the and was measured by the Lowry technique (Bio-Rad protein assay, Bio-Rad).

Evaluation of Serum Antibody Responses to *H. hepaticus*

At necropsy, blood was collected via cardiac puncture from CO2 asphyxiated mice. Blood was centrifuged at 10,000 rpm for 10 minutes, and the serum preserved with 15mM sodium azide. An enzyme-linked immunosorbent assay (ELISA) was used to detect *H. hepaticus*-specific serum IgG and IgA. Nunc Polysorb 96-well plates (Nalge Nunc International, Rochester, NY) were coated with 100 μL of a 10-μg/ml concentration of *H. hepaticus* protein in carbonate buffer (pH 9.6) overnight at 4°C. Plates were blocked with 1% bovine serum albumin in phosphate-buffered saline. Serum was diluted 1/100 and biotinylated secondary antibodies included goat anti-mouse IgG1 (Sigma) diluted 1/5,000 and goat anti-mouse IgG2c (Southern Biotech, Birmingham, AL) diluted 1/1,000. Incubation with extravidin-peroxidase (Sigma) at a dilution of 1/1000 was followed by incubation with 2,2'-Azine-di[3-ethylbenzthiazoline sulfonate] (ABTS) horseradish peroxidase substrate (Pierce) for color development. Optical density at 405nm was recorded by an ELISA plate reader (VERSAmax; Molecular Devices, Sunnyvale, CA).

DTH Response

To measure the development of delayed type hypersensitivity, mice were given 10 μg of *H. hepaticus* antigen in a volume of 50 μL (prepared in B-PER I Reagent as above and diluted in PBS) by injection into the hind footpad. The opposite footpad was injected with PBS alone. Twenty-four hours after injection, footpad thickness was measured with a dial thickness gauge (Mitutoyo Corporation, Kawasaki, Kanagawa, Japan), and the difference in thickness between the control and antigen-treated footpad recorded.

Statistical Analysis

Statistical analysis was performed using the JMP statistical package (SAS, Cary, NC). Categorical inflammation scores were compared by the nonparametric Wilcoxon rank-sum test with statistical significance set to a P value <0.05. ELISA and DTH data were analyzed by analysis of variance. The Tukey-Kramer HSD test was used to identify groups with significantly different means with an alpha level set to 0.05.

Short Term IBD Study

Published in Infection and Immunity as "In vitro and in vivo characterization of *Helicobacter hepaticus* cytolethal distending toxin mutants." 2004 May; 72 (5):2521-7.

Rationale:

H. hepaticus has been shown to cause inflammatory bowel disease in immune altered mice. Specific-pathogen-free IL-10–/– mice develop severe typhlocolitis when they are infected with *H. hepaticus*. In order to test the role of CDT in the pathogenesis of disease caused *by H. hepaticus*, isogenic CDT mutants were created and tested for the ability to cause IBD in C57BL/6 IL-10 -/- mice. Because they lack IL-10, a major signal in the downregulation of the TH-1 response, and upregulation of the TH-2 response pathway, these mice are biased towards a TH-1 inflammatory response. This response is analogous to that seen in Crohn's disease in humans. The goal of this experiment was to establish a link between the presence of a function CDT holotoxin and the ability of *H. hepaticus* to cause gastrointestinal disease in a murine model.

Results:

CDT Influences the Ability of *H. hepaticus* to Trigger Colitis

In this study, specific-pathogen-free (possessing no known *Helicobacter spp.)* C57BL/6 IL-10 knockout mice, were orally challenged with either wild-type *H. hepaticus* 3B1, the CDT positive isogenic mutant , or the CDT negative mutant. To serve

as a negative control, a group of animals was gavaged with sterile trypticase soy broth. The colonization status of these animals was monitored via collection of fecal pellets by culture, DNA extraction and PCR. Confirmation of the presence of the isogenic *H. hepaticus* CDT mutants was accomplished by PCR with primers spanning regions of the CDT locus. PCR performed on these mutants produced a larger amplicon when compared to wildtype *H. hepaticus*. The difference in amplicon size corresponds with the insertion of the transposon in and around the genes.

At six weeks post infection , animals were sacrificed and a necropsy was performed. At this timepoint, all of the animals that received *H. hepaticus* remained colonized as determined by PCR and culture. In all cases, only the specific challenge strain was recovered from infected animals. By 6 weeks post infection, none of the control animals developed significant typhlocolitis (See Figure 4A, Fig. 5). One control animal had a small focus of chronic inflammatory cells at the ileocecal junction, a lesion that can occur spontaneously in uninoculated animals. All of the IL 10 $^{-/-}$ animals in the positive control group (infected with wildtype *H. hepaticus*) developed lesions, and pathology of inflammatory bowel disease. These animals developed extensive mucosal hyperplasia, accompanied by mucosal and submucosal inflammation (See Figure 4B, Fig. 5). IL-10–/– mice infected with the transposon-marked CDT-producing isogenic mutant 3B1::Tn16 also developed typhlocolitis with a severity equivalent to that of mice infected with wild-type *H. hepaticus* 3B1 (See Figure 4C, Fig. 5). IL-10–/– mice that were

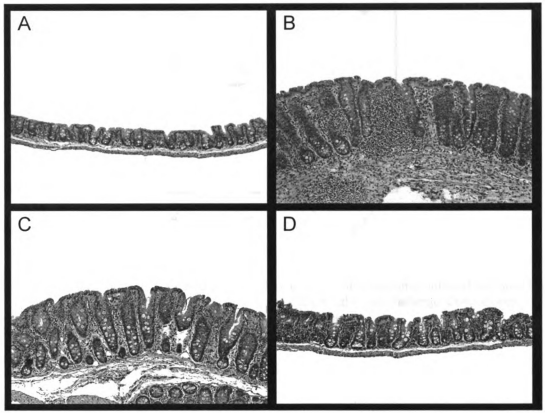

Figure 4. Histolopathologic lesions in the cecae of IL-10-/- mice 8 months after challenge with isogenic strains of *H. hepaticus*. IL-10-/- mice were sham infected (A), infected with wildtype *H. hepaticus* strain 3B1 (B), the CDT-positive mutant 3B1::Tn16 (C), or the CDT-negative mutant 3B1::Tn20 (D). Mice challenged with CDT-producing strains exhibited marked inflammation and hyperplasia. Mice challenged with the CDT-negative mutant, which was cleared by 4 months after infection, developed diminished disease.

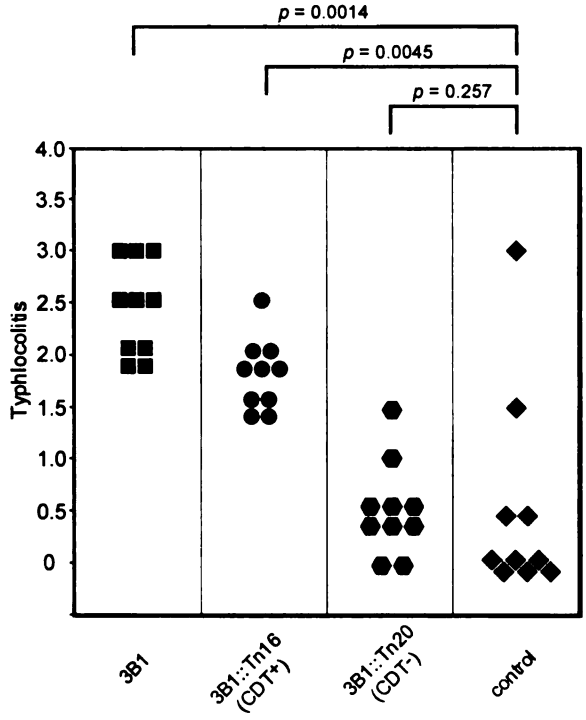

Figure 5. Categorical inflammation scores for uninfected IL-10-/- mice and mice infected with wild type *H. hepaticus* strain 3B1 and the isogenic mutant strains eight months after challenge. Comparisons between groups were performed using the Wilcoxon rank sum test.

infected with the CDT-negative mutant 3B1::Tn20, however, exhibited significantly less severe typhlocolitis than mice infected with CDT-producing *H. hepaticus* strains. Although all mice were infected with the mutant *H. hepaticus*, one mouse was histologically normal, and the majority of animals exhibited only mild, patchy mucosal inflammation (See Fig. 4D, Fig. 5). Although two mice infected with 3B1::Tn20 did develop diffuse mucosal inflammation, none of the mice infected with the CDT-negative mutant developed the submucosal inflammation encountered in a subset of the mice infected with CDT-positive strains. Mesenteric perivasculitis was encountered in infected animals with the most severe inflammation but never in uninfected controls.

Discussion:

The collected data indicates that *H. hepaticus* CDT may play a significant role in the development of inflammatory bowel disease by infected immune-altered mice. Isogenic CDT mutants developed less severe disease than mutants infected with the wild-type strain of *H. hepaticus*. There was a drastic quantitative and qualitative difference in the nature of the typhlocolitis. None of the mice infected with the CDT negative mutants developed significant submucosal inflammation, unlike the CDT positive strains.

Cytolethal distending toxin activity in *H. hepaticus* is not required to initiate inflammatory bowel disease in IL-10-deficient mice, but influences disease severity. Transposon mutants that lie in the coding sequence for the *cdtABC* gene cluster were disruptive enough to eliminate cytotoxic activity. The presence of the Chloramphenicol resistance marker does not effect ability of the isogenic mutants to cause either IBD or hepatitis. The CDT negative mutant is unable to colonize wildtype mice after 4 months

post infection, suggesting that CDT may be required for long-term colonization of wildtype mice.

Long Term IBD and Gastrointestinal Cancer Study

Rationale:

After exploring the roles of CDT in the etiopathogenesis of murine IBD in a short term study and hepatic disease in a long term study, role of CDT in a long term IBD study needed to be elucidated. Since the CDT negative mutant was unable to persist in A/JCr mice with wildtype immune function, the question was raised as to whether the mutant could persist in immune altered C57BL/6 IL-10 -/- mice. In this study, mice were infected with wildtype *H. hepaticus* as well as the isogenic mutants over a 9 month study to determine the role of CDT in a long term IBD and gastrointestinal cancer study.

Results

Our previous results show that infection of IL-$10^{-/-}$ mice with the CDT-deficient *H. hepaticus* mutant is associated with decreased IBD pathology six weeks after challenge compared to animals infected with wild-type *H. hepaticus* (31). Both the CDT-deficient mutant and wild-type *H. hepaticus* were easily detectable by culture and PCR at the end of this six-week infection study.

To determine the role of CDT in the development of long-term sequelae of *H. hepaticus* infection including colonic neoplasia, additional infection studies were initiated using isogenic CDT mutants. The *H. hepaticus* mutant 3B1::Tn20 is deficient in CDT-production due to a transposon insertion in *cdtA*. 3B1::Tn16 is an isogenic control mutant that has a transposon insertion downstream of *cdtC* in a presumed intergenic region (31).

Four to six week-old female C57BL/6 IL-$10^{-/-}$ mice were infected with wild-type *H. hepaticus* strain 3B1 and the two isogenic mutant strains. An equal number of control mice were sham-infected with sterile culture broth. There were ten animals in each experimental group. Animals in all experimental groups were monitored for *H. hepaticus* colonization by *H. hepaticus*-specific PCR amplification of DNA isolated fresh fecal pellets and at certain time points, by selective culture for *H. hepaticus* from feces.

C57BL/6 IL-$10^{-/-}$ mice were initially colonized by wild-type *H. hepaticus* 3B1 and by both isogenic mutants. Wild-type *H. hepaticus* and the CDT-positive isogenic mutant 3B1::Tn16 colonized both strains of mice for the entire duration of each experiment. Conversely, although the CDT-deficient mutant 3B1::Tn20 was detectable at 45 days post infection (p.i.) in IL-$10^{-/-}$ animals, this mutant was not found by PCR or culture at 115 days p.i. All mice challenged with 3B1::Tn20 remained negative for *H. hepaticus* for the remainder of each experiment. At all times where colonization was assessed by both culture and PCR for *H. hepaticus*, there was concordance between the two methods.

Mice that clear infection develop minimal typhlocolitis

Histological examination was performed of the ileocecocolic junction of the IL-$10^{-/-}$ mice challenged with wild-type 3B1 and the two isogenic mutants 251 days post infection. This time point was 190 days after the CDT-deficient mutant 3B1::Tn20 was last detected in the feces of infected animals, and 136 days after the animals were first shown to be free of *H. hepaticus*. Of the forty animals in this experiment, three had to be sacrificed prior to this time. One animal infected with 3B1::Tn16 developed rectal prolapse 59 days p.i., and another 3B1::Tn16-infected animal had to be euthanized due to

severe dermatitis 160 days p.i. One animal infected with wild-type 3B1 developed rectal prolapse 130 days p.i.

Nine of the ten control animals had no or minimal evidence of inflammation at the ileocecocolic junction at the time of necropsy. One of the control animals had significant inflammation and hyperplasia. Feces and tissue from this animal were examined carefully for *H. hepaticus* infection by culture and PCR, and *H. hepaticus* was not detected in this outlier.

Animals with infected with either wild-type *H. hepaticus* or the CDT-positive mutant 3B1::Tn16 developed moderate to severe typhlocolitis. These animals developed marked mucosal hyperplasia with infiltration of the lamina propria with chronic inflammatory cells. This disease was significantly different compared to uninfected controls. IL-$10^{-/-}$ mice that were initially infected with the CDT-deficient mutant 3B1::Tn20 but eventually cleared the infection between 61 and 115 days p.i. had minimal signs of inflammation. Two animals had no visible inflammatory infiltrates, while the majority of the remaining animals only had small, scattered aggregates of chronic inflammatory cells with minimal hyperplasia. The scores of the animals infected with the CDT-deficient mutant 3B1::Tn20 were not significantly different from the scores from uninfected controls.

The CDT positive mutant,3B1::Tn16 and CDT negative mutant, 3B1::Tn20 were both able to effectively colonize C57BL/6 IL-10 -/- mice, and were detectable for the first sixteen weeks of the study by PCR of a fecal DNA prep. At 36 weeks post- infection, none of the animals in the control group showed any signs of typhlocolitis. All of the IL $10^{-/-}$ animals in the positive control group (infected with wildtype *H. hepaticus*)

developed lesions, and pathology of inflammatory bowel disease. These animals developed extensive mucosal hyperplasia, accompanied by mucosal and submucosal inflammation, and some individuals developed adenocarcinoma (See Fig. 6). IL 10 $^{-/-}$ mice infected with the transposon marked CDT mutant 3B1::Tn16 also developed severe typhlocolitis, equivalent to that seen in the wild-type infection. Mice infected with the 3B1::Tn20 mutant developed disease, however, it was much less severe than either of the CDT (+) infected animals. 3B1::Tn20 infected mice also exhibited a significantly decreased amount of mucosal hyperplasia than 3B1, or 3B1::Tn16. The 3B1::Tn20 mutant was unable to colonize IL-10 -/- mice after 16 weeks post infection. None of the animals infected with 3B1::Tn20 exhibit tumor formation in the cecum or colon.

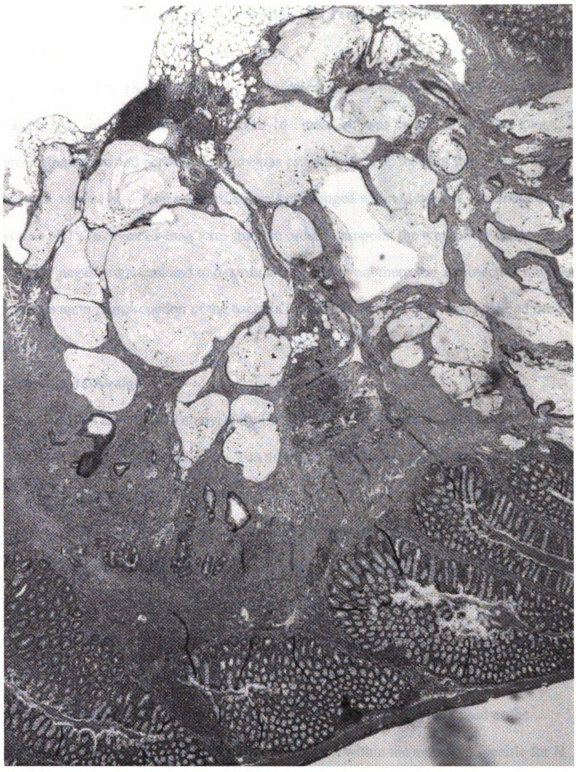

Figure 6. Mucinous adinocarcimona in the proximal colon of a C57BL/6 IL-10 knockout mouse infected with wildtype *H. hepaticus* at 8 months post infection. Characteristic glandular invasion into the submuscosa was observed.

Long Term Colonization and Persistence Study

Rationale:

Since 3B1::Tn20, the isogenic *H. hepaticus* mutant that lacked CDT production was initially able to colonize C57BL/6 IL-$10^{-/-}$ mice, but was unable to persist, a long term colonization and persistence study was established. The focus of this study was to determine the colonization status of animals challenged with wildtype *H. hepaticus* as well as 3B1::Tn20 over a long term study in order to pinpoint the window of clearance of the CDT negative mutant, and to determine whether or not there was a protective immune response upon reintroduction of the bacteria to animals that had previously cleared the infection.

Experimental Design

For this longitudinal study, sixty C57BL/6 IL 10 -/- were housed in cages ranging from three to five animals per cage. Ten mice served as uninfected controls and ten were challenged with wildtype *H. hepaticus.* Forty mice were challenged with the CDT deficient strain, 3B1::Tn20. Colonization status was monitored using fecal samples taken aseptically approximately every three weeks throughout the duration of the study. Each cage was considered a unit of infection because of the coprophagic nature of the mice. Nested PCR assays were used to determine colonization status (See Fig. 7). A primary PCR was performed with primers C05/C97 that amplify a region of the small 16S rRNA gene of all known *Helicobacter* species. The product of this assay was used as the template for a secondary PCR which bind to a region within the amplicon specific for *H. hepaticus.* The nested PCR has the ability to detect between 25-50 genome equivalents of *H. hepaticus* DNA in approximately 100 ng DNA prepared from a fecal pellet.

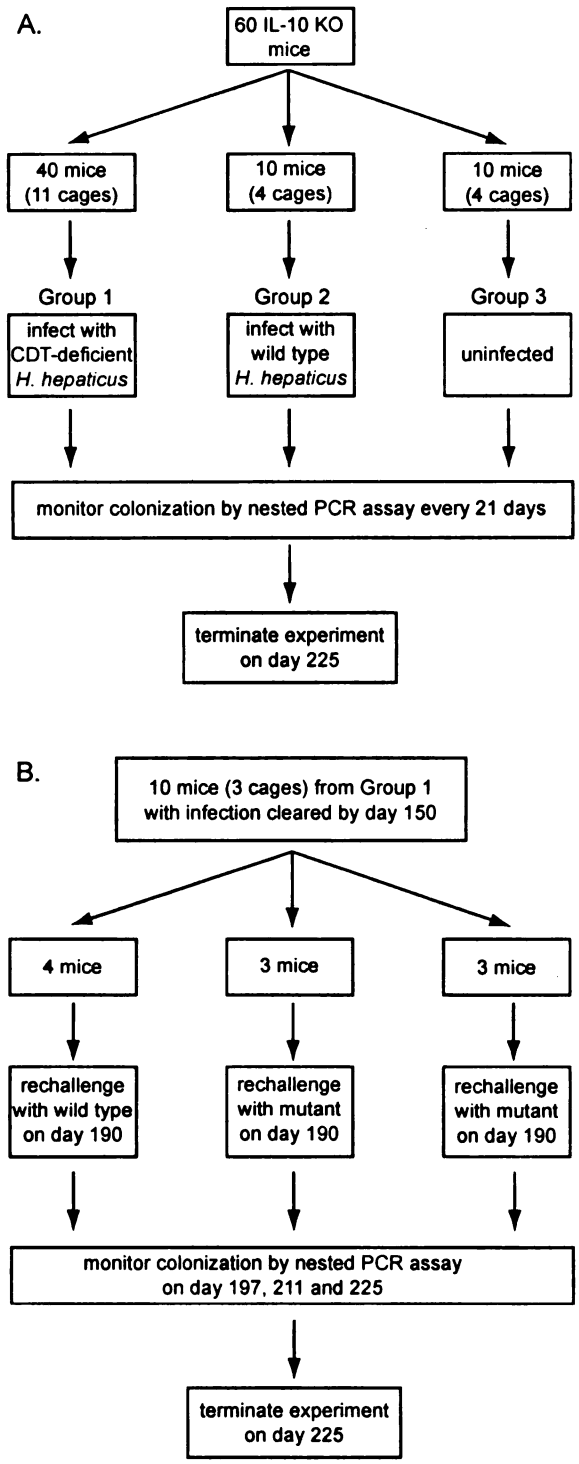

Figure 7. Design of longitudinal infection study to follow the loss of gastrointestinal colonization by a CDT-deficient *H. hepaticus* strain. A) Overall experimental design. 60 IL-10-/- animals were divided into three groups. Group 1 was infected with the CDT deficient *H. hepaticus* strain, group 2 infected with wild type *H. hepaticus* and group 3 was sham-infected with sterile culture broth. Colonization was monitored by a nested *H. hepaticus*-specific PCR assay. B) Design of nested experiment to test if clearance of the CDT-deficient *H. hepaticus* mutant was associated with development of protection against reinfection. Ten mice originally challenged with the CDT-deficient mutant were rechallenged with *H. hepaticus* at least 40 days after clearance of the mutant.

Results

All mice were initially colonized by wildtype *H. hepaticus*, as well as the CDT deficient strain. Wildtype *H. hepaticus* was able to persist in the gastrointestinal tract, and was detectable in the feces for the duration of the experiment. As indicated by our previous studies, the CDT deficient mutant was not able to persist. At 75 days post infection, all samples were positive for *H. hepaticus* colonization by primary PCR. However, an increasing fraction of the cages were negative by primary PCR from this timpepoint on. All cages challenged with the CDT deficient mutant remained positive by the more sensitive secondary portion of the nested PCR until 117 days post infection (See Table 1). At subsequent timepoints, colonization status by the secondary PCR assay followed the form of the primary PCR, as more cages tested negative for the presence of *H. hepaticus*. This demonstrated that the CDT negative mutant was unable to maintain persistence in the mouse gastrointestinal tract for greater than four months post infection (See Fig. 8).

CDT plays a significant role in the development and severity of inflammatory bowel disease in *H. hepaticus* infected IL-10 knockout mice. Additionally, the presence of a functional CDT holotoxin affects colonization, and is necessary for long-term persistence of *H. hepaticus* in immune-altered mice.

Antibody Responses Against *H. hepaticus* in IL-10$^{-/-}$ Mice

Mice infected with *H. hepaticus* develop a significant specific antibody response to *H. hepaticus*. None of the sera from uninfected C57BL/6 IL-10$^{-/-}$ animals contained antibody reactive to *H. hepaticus* antigens measured by ELISA. All animals infected with wild-type *H. hepaticus* and the CDT deficient isogenic mutant developed a robust

Challenge Strain	PCR (culture)					
	9d. p.i.	32d. p.i.	45d. p.i.	61 p.i.	115d. p.i.	251d. p.i
Wild-type	+	+ (+)	+ (+)	+	+ (+)	+ (+)
3B1::Tn16 (CDT+)	+	+ (+)	+ (+)	+	+ (+)	+ (+)
3B1::Tn20 (CDT-)	+	+ (+)	+ (+)	+	- (-)	- (-)
uninfected	-	- (-)	- (-)	-	- (-)	- (-)

Table 1. Temporal monitoring of the colonization of IL-10-/- mice with *H. hepaticus* by *H. hepaticus*-specific PCR and culture.

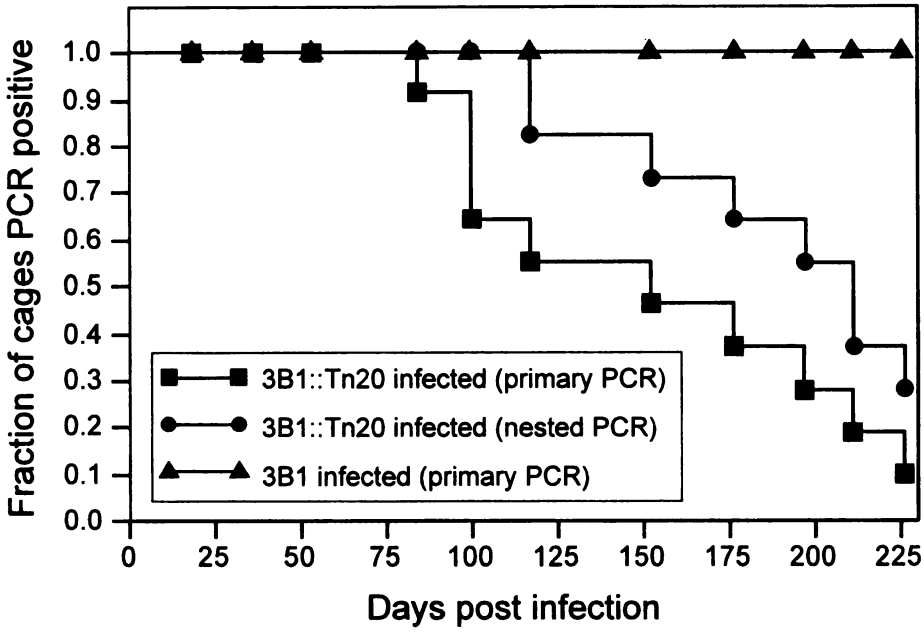

Figure 8. Monitoring the loss of colonization with a CDT-deficient *H. hepaticus* mutant using a nested PCR assay. *H. hepaticus* could be detected in the feces of animals challenged with wild type *H. hepaticus* for the entire 225 duration of the experiment. There was progressive loss of colonization with the CDT-deficient mutant starting 75 days after infection, as judged by the primary stage of the PCR assay, and starting at day 125, as judged by the nested assay.

anti-*H. hepaticus* serum Th2 associated IgG1 and TH1 associated IgG2c specific humoral immune response (See Fig. 9A,B). Serum samples collected at 225 days post infection (the termination point of the experiment), animals infected with wildtype *H. hepaticus* developed a greater IgG response than animals infected with the CDT deficient mutant. Rechallenge of 3B1::Tn20 infected animals that had subsequently cleared infection showed a boosted IgG2c response when compared to the uninfected animals, or animals that cleared and were not rechallenged. The IgG2c response ranged from approximately 2.5 fold higher in 3B1 infect animals, to approximately 10 fold higher in rechalleneged animals (See Fig. 9B).

Delayed type hypersensitivity (DTH) testing was used to measure the development of a cellular immune response specific to *H. hepaticus*. Ten micrograms of a BPER detergent protein extract of *H. hepaticus* in 50 microliters of PBS was injected into the right hind footpad of the mice. The left hind footpad was injected with PBS containing a diluted amount of BPER reagent to serve as a negative control. The difference in footpad thickness was measured using a dial thickness gauge at 24 and 48 hours. Mice infected with wildtype *H. hepaticus,* as well as the CDT deficient mutant, showed a significant DTH response to *H. hepaticus* antigen when compared to uninfected control mice. Mice originally infected with wildtype *H. hepaticus* showed a greater DTH than those that were originally infected with the CDT deficient mutant. There appeared to be a booster effect similar to that seen in the ELISA in mice that cleared infection and were rechallenged when compared to those that had cleared, but were not rechallenged (See Fig. 9C).

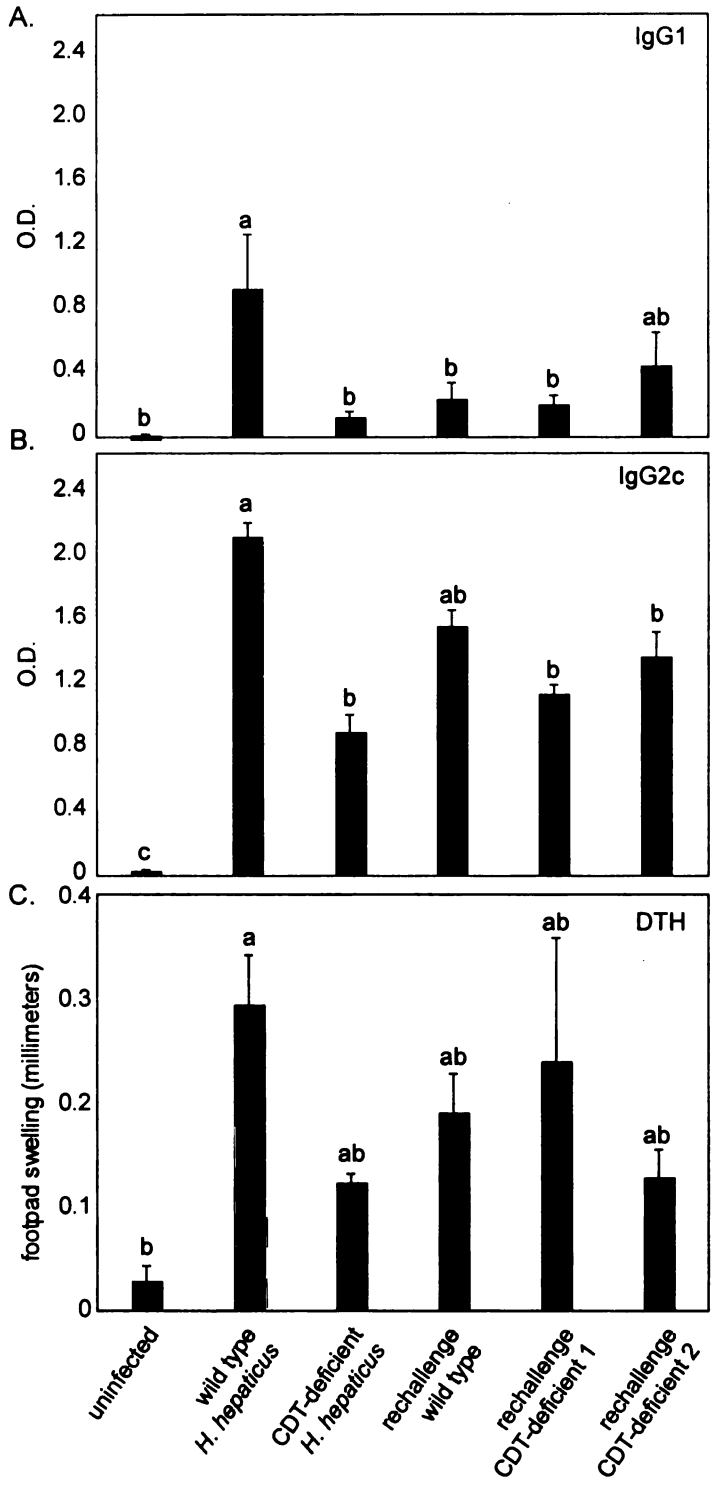

Figure 9. Development of IgG1 (A), IgG2c (B) antibody responses and delayed type hypersensitivity (C) to *H. hepaticus*. All animals infected with either wildtype *H. hepaticus* or the CDT deficient mutant developed *H. hepaticus*-specific IgG1 and IgG2c responses with a > 2:1 ratio of IgG2c: IgG1 consistent with a TH1 response. For each panel, groups not connected by the same letter were significantly different by Tukey-Kramer HSD with an alpha level set at 0.05. O.D. optical density.

Mice that Clear Infection are Protected from Rechallenge

Once it was established that C57BL/6 IL 10 -/- mice were able to clear infection of the CDT negative strain of *H. hepaticus*, we wished to determine if clearance was coupled with a protective immunity. Three cages of the mice that were initially challenged with 3B1::Tn20, but had cleared infection were rechallenged. One cage containing 4 mice were rechallenged with wildtype *H. hepaticus* while two cages (3 mice each) were rechallenged with the CDT negative isogenic mutant. One cage that had cleared infection was not rechallenged to serve as a control. Colonization status was monitored by nested PCR as previously described.

One cage of the infected animals rechallenged with 3B::Tn20 showed no detectable levels of *H. hepaticus* at 7 and 21 days after the rechallenge by both the primary and secondary PCR assays. The other cage of mice rechallenged with 3B1::Tn20 was positive by both primary and the secondary PCR on day 7, but was negative by primary PCR on day 21. Animals rechallenged with wildtype *H. hepaticus* were negative by primary PCR on days 7 and 21, but were positive by the nested PCR (See Table 2).

At day 35 after rechallenge, fecal pellets were collected from each individual mouse to determine if colonization status varied within each cage. Due to the coprophagic nature of the mice, it was hypothesized that all animals within a cage would have the same colonization status. Surprisingly, the PCR assay on the individual samples revealed that there was animal to animal variation within a cage, as well as in different cages subject to the same treatments (See Table 2).

	Rechallenge Strain									
	wild-type *H. hepaticus*				3B1::Tn20 cage #1			3B1::Tn20 cage #2		
Primary PCR	-	+	-	-	-	+	+	-	-	-
Nested PCR	-	+	-	-	+	+	+	-	+	-

Table 2 Assessment of colonization status in mice that cleared previous infection with the CDT-deficient mutant 3B1::Tn20, 35 days after re-challenge with wild type or mutant *H. hepaticus*

Three of the four mice rechallenged with wildtype *H. hepaticus* showed clearance and were negative by both primary and secondary PCR at 35 days after the rechallenge. The one animal in this cage that remained positive was shedding a relatively large amount of *H. hepaticus* as its levels were detectable by both primary and secondary PCR. *H. hepaticus* was cultured from the feces of this animal, and was confirmed by PCR to be wildtype *H. hepaticus,* rather than the 3B1::Tn20 by which it was originally challenged (See Table 2).

The two cages of mice rechallenged with the CDT negative mutant showed varying results. In one cage, there was minimal evidence of clearance, as two of the animals were positive by both primary and nested PCR. The other animal was negative by primary PCR, but positive by secondary PCR. In the other cage of animals rechallenged with the CDT deficient strain, two of the three animals were negative for *H. hepaticus* by both primary and nested PCR, while the other animal was negative by primary, but positive by secondary PCR at day 35.

Long-Term Hepatitis / Hepatocellular Cancer

Rationale:

H. hepaticus has been linked to chronic active hepatitis and hepatocellular carcinoma in wildtype A/JCr mice. Since strains lacking a functional CDT holotoxin caused less disease in a murine IBD model, we wished to test the role of CDT in *H. hepaticus* induced liver disease. Isogenic CDT mutants were tested for the ability to cause hepatitis and hepatocellular cancer in A/JCr mice in a long-term study. The goal of this experiment was to establish a link between the presence of a function CDT holotoxin and chronic hepatitis and hepatocarcinoma.

Results

In the hepatitis study, wildtype and the CDT positive mutant 3B1::Tn16 were able to colonize A/J mice in a long-term study and were detectable at 18 months post infection via PCR. The CDT negative mutant, 3B1::Tn20 was detectable by PCR at one month and two months post infection in the A/J mice, but was undetectable in DNA from a fecal prep at 4 months, 6 months, 11 months post infection and throughout the duration of the study. The animals infected with CDT positive strains (3B1 and 3B1::Tn16) developed hepatitis including a marked increase in inflammatory infiltrates located in the lobules near the central vein in the portal tracts, as well as calcification in the parenchyma (See Fig. 10 B, C). The control animals and those challenged with the CDT negative strain, 3B1::Tn20, showed little to no hepatitis (See Fig. 10 A, D). *Helicobacter* was observed in tissue via Steiner stain and confirmed by PCR and recovered by plating on selective

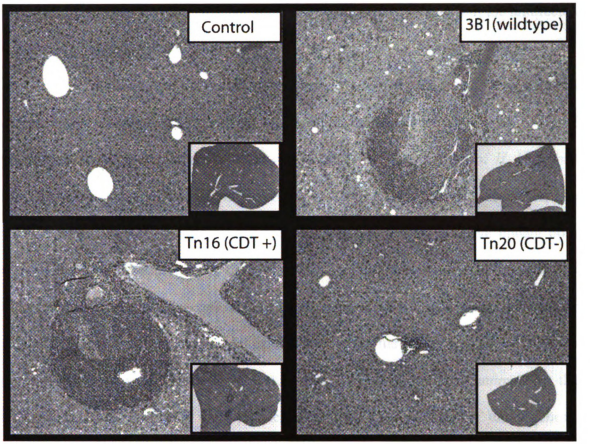

Figure 10. Representative histopathology of hepatitis with involvement of portal areas. Progressive chronic inflammation centered around veins and bile ductules. Inflammation occured throughout the liver and was associated with biliary epithelial hyperplasia and dysplasia. Inflammatory infiltrate consists primarily of lympoctyes, including plasma cells, eosinophils and neutrophils and involves areas of parenchyma. The 3B1 and 3B1::Tn16 challenged mice showed a much more severe inflammation when compared to the control and 3B1::Tn20.

media in the CDT positive strains, but was undetectable by any method in the uninfected control and the Tn20 infected animals (See Fig. 11).

At 18 months, the termination point of the experiment, the livers were extracted and examined histologically for evidence of hepatitis and hepatocellular cancer. None of the control animals, or animals infected with the CDT deficient strain of *H. hepaticus* showed marked inflammation of the liver or hepatocellular tumorigenesis. However, The animals infected with wildtype *H. hepaticus* as well as the CDT positive mutant both showed marked inflammation of the liver. Over half of the animals sampled at this timepoint from the two groups CDT positive groups developed noticeable tumor formation grossly. Upon histological examination, all of the wildtype and CDT positive mutant had developed adenomas or carcinomas, while none of the animals in the uninfected control group, or the CDT negative group showed signs of tumorigenesis (See Fig. 12).

Discussion

The *in vitro* effects of CDT on cells are most likely different than the effect seen *in vivo*. Cell cycle arrest is seen in the G2/M transition of the cell cycle after exposure to high levels of CDT due to the cell's check point control. This leads to distension, nuclear fragmentation and eventually cell death. It is likely that the damage caused by CDT in this case overwhelms the checkpoint control. Low level exposure to CDT is often insufficient to see cytotoxic effects, but it is likely that these cells that are able to bypass the checkpoint by repairing damage have a greater likelihood of mutations that lead to tumorigenesis. DNA damage caused by exposure to CDT should lead to an increase in

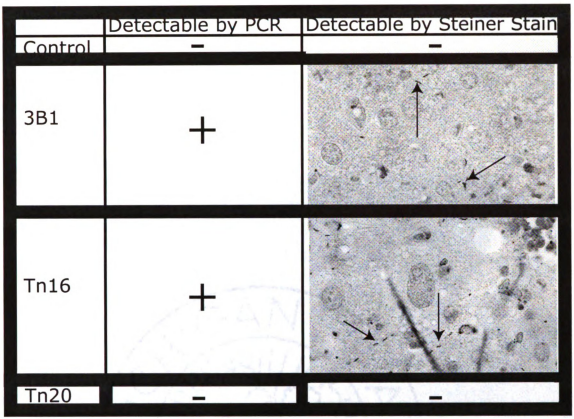

Figure 11. At 4 months post infection, two mice per cage were sacrificed and their livers removed. DNA was extracted from liver tissue and *H. hepaticus* specific PCR was performed. Animals infected with the CDT positive mutants showed a positive test for *H. hepaticus* by PCR and were also visible in the tissue by the silver staining procedure, steiner stain. Control animals were negative both by PCR and steiner stain. Surprisingly, the CDT negative mutant was unable to colonize and was not detectable by PCR or steiner stain.

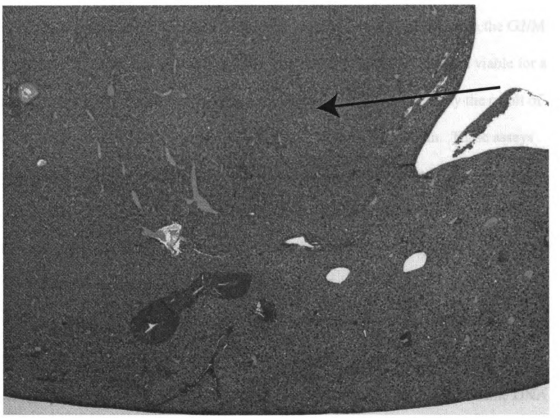

Figure 12. Histological section of the left lobe from the liver of an animal infected with the CDT positive mutant, 3B1::Tn16. The large adenoma, indicated by the arrow, could be observed grossly at necropsy. Upon microscopic examination, there was evidence of a large inflammatory infiltrate in the left lobe, but not in the tumor itself. Also, a lack of bile duct formation was observed, a trademark of liver tumorigenesis.

tumorigenesis, as well as an increase in cytotoxicity, depending on the level of exposure to the toxin.

Cytolethal distending toxin is a potent virulence factor produced by a number of pathogenic bacteria in order to manipulate host cells. The active subunit of the toxin produces double strand breaks in the DNA of intoxicated eukaryotic cells. The effects of this damage elicit a checkpoint control response, leading to cell cycle arrest in the G2/M transition. Cells that are unable to repair the damage caused by CDT remain viable for a period of time, before undergoing apoptosis. This cell death is most likely the result of catastrophic amounts of DNA damage conferred by high levels of toxin. These assays are reproducible *in vitro,* but the level of exposure to the toxin *in vivo* is much tougher to gauge. It is likely that low level exposure, perhaps as little as one molecule of toxin, is sufficient to cause double strand breaks in the nucleus. Low-level exposure is unlikely to overwhelm the host cell repair mechanisms, allowing them to function normally. These cells will be able to pass the checkpoint, and continue through the cell cycle. This DNA damage caused by CDT is similar to the effects of ionizing radiation, a known carcinogen. As a result, treatment of eukaryotic cells with CDT employs the same DNA repair mechanisms as ionizing radiation. Inefficient or error prone repair of chromosomal aberrations caused by DNA damaging agents has been implicated in the formation of cancer cells.

We are unable to determine if the tumorigenesis observed in infected animals is a result of DNA damage, or simply a byproduct of long-term inflammation. In some studies, reactive oxygen species released as a result of chronic infections have been shown to cause tumor formation.

General Discussion

The chronic nature of infection is a hallmark of *Helicobacter* infections. While this feature may be of minimal significance to the host in some cases, chronic *Helicobacter* infections and the associated long-term inflammation can lead to the development of neoplastic disease. Bacteria that are able to cause persistent infections have developed varied strategies to evade immune surveillance (20, 29). *H. pylori* produces the virulence factor vacuolating cytotoxin A (VacA) which has a theorized role in the evasion of immune surveillance by *H. pylori* , thus allowing it to chronically colonize the gastric mucosa (21). VacA has been shown to inhibit T cell activation, and thus may interfere with the generation of a protective immune response by the host (1, 15). While *H. hepaticus* does not possess a homologue of *vacA,* the structural gene for VacA, it is hypothesized that another toxin may play a similar role in this organism. Cytolethal distending toxin, which has no known homologues in *H. pylori*, may serve as an immunoregulatory toxin with similar function in *H. hepaticus*. CDT has been shown to induce apoptosis in primary human peripheral blood mononuclear cells and cultured T-cell lines (24, 30, 32). In addition to a direct effect on T-cells, CDT may be able to influence the host immune response pathways by interfering with antigen presenting cells. It was recently shown that primary human macrophages and dendritic cells treated with CDT showed a decreased efficiency in cytokine production and the ability to stimulation of T-cell proliferation.

In three independent studies, we provide evidence that CDT is necessary for long-term colonization of the murine gastrointestinal tract by *H. hepaticus*. A CDT-deficient

isogenic mutant of *H. hepaticus* is able to maintain colonization of IL-10-/- mice for at least 61 days after its introduction to the animal, but is subsequently cleared by 115 days following experimental infection. Questions may arise about the fitness of the organism as a result of producing an extra protein, chloramphenicol acetyl-transferase, at relatively high levels. This is however been remedied by construction of an isogenic mutant that retains a functional CDT gene cluster. The inability of the CDT-deficient mutant to persist is not an effect of the construction of the transposon mutant since the CDT positive mutant maintained CDT expression and was able to maintain persistent colonization. This suggests that IL-10- /- mice infected with the CDT-deficient mutant are able to mount an immune response that results in clearance of the organism, a phenomenon that is not apparent in animals challenged with wildtype *H. hepaticus* or the CDT positive mutant.

At the end of the experiment (225 days after initial challenge with *H. hepaticus*) the magnitude of *H. hepaticus*-specific humoral and cellular immune responses was greater in animals infected with wild type *H. hepaticus*, as judged by ELISA reactivity and development of DTH. The most likely explanation for this is that these responses were measured at a time well after clearance of the CDT deficient mutant, and the loss of colonization was responsible for waning of immunity. Our results are in general agreement with Ge and colleagues, who reported that an independent CDT deficient *H. hepaticus* mutant was unable to maintain persistent colonization of outbred Swiss Webster mice (14). In their study, mice infected with either wild type *H. hepaticus* or their CDT deficient mutant developed significant *H. hepaticus*-specific ELISA reactivity compared to uninfected controls. However they noted that mice infected with the CDT

deficient mutant had decreased reactivity compared to animals infected with wild type. This may reflect the time that passed between clearance of the organism and the collection of serum at the end of the experiment.

In the current study, we have extended the finding that a CDT deficient *H. hepaticus* mutant is unable to maintain long term colonization of the murine gut by showing that animals that clear infection are provided with a degree of protection against reinfection. Initial infection with the CDT deficient mutant 3B1::Tn20 is associated, in a subset of individuals, with the development of a protective immune response against re-challenge with either 3B1::Tn20 or wild- type *H. hepaticus*. The development protective immunity against wild-type *H. hepaticus* is significant. It is possible that 3B1::Tn20 has a defect (perhaps unrelated to lack of CDT) that is responsible for the long-term colonization defect. This defect may be amplified by rechallenge of an animal that has already encountered the organism, and thus loss of the organism upon rechallenge is not necessarily reflective of protective immunity. However, the fact that animals that have lost colonization with 3B1::Tn20 rapidly clear rechallenge with wild-type *H. hepaticus* provides much stronger evidence that this is due to the generation of protective immunity. In addition to an essential role in maintaining long-term intestinal colonization, CDT also appears to have a proinflammatory role during *H. hepaticus* infection of IL-10-/- mice. The mechanism of *H. hepaticus* clearance is yet to be determined. It remains unclear if the lack of persistence in the mutant is due to an increase in the ability of the animal's immune response to clear the pathogen, or to the inability of the organism to compete with the resident microbiota due to a lack of fitness. CDT has been shown to have an immunomodulatory activity (28), and it is possible that the absence of a functional

holotoxin in the mutant renders it more susceptible to the host immune response. It is likely that *H. hepaticus* CDT alters the nature and/or specificity of the host immune response, allowing persistence.

In an earlier study, we noted that six weeks after infection, IL-10-/- mice challenged with the CDT deficient *H. hepaticus* mutant developed significantly less typhlocolitis than animals infected with wild- type *H. hepaticus* despite similar levels of colonization (42). In the current study, we demonstrate that eventual clearance of the CDT deficient *H. hepaticus* mutant results in almost complete resolution of typhlocolitis. The combined roles of CDT, having both immunomodulatory activity (allowing escape from immune surveillance and chronic colonization) and also proinflammatory activity (leading to sustained typhlocolitis) again mirrors the VacA toxin of *H. pylori* (21). It remains to be determined if similar roles will be found for other bacteria that elaborate CDT.

IgG interactions stimulate ingestion of cells and foreign particles by macrophages and neutrophils. A recent study by Xu et al states that *H. ducreyi* CDT induces the apoptosis of dendritic cells and hampers both the induction of cytokine production by antigen presenting cells (APCs) and the ability of APCs to elicit T-cell activation. The modulation of the host immune system by wildtype *H. hepaticus* CDT would allow for greater persistence in spite of the presence of increasing IgG levels. The inability of the mutant to produce a functional CDT holotoxin, and therefore, alter the immune response, leaves it more susceptible to immune clearance.

Mice infected with the CDT (-) strain also showed a delay in the rise of the total IgG in response to *H. hepaticus* than wildtype infected animals. The delay could be a

result in the inability of the CDT negative mutant to colonize as rapidly as wildtype because it cannot modulate the immune response. The total IgG response to *H. hepaticus* peaks at approximately 8 weeks, and appears to level off, maintaining a relatively constant level. By altering the function of APCs, wildtype *H. hepaticus* is able to thrive in the presence of an elevated IgG response, whereas mutants lacking CDT are unable to persist, leading to lower levels, and eventually clearance of the organism. Mice infected with the CDT negative strain also showed lower levels of total IgG in response to *H. hepaticus* than wildtype infected animals. Although the results to date are not statistically significant, there appears to be a trend to the decrease in colonization of 3B1::Tn20 infected animals. Whether this is due to an increase in the ability of the animal to clear the pathogen, or to the inability of the organism to persist is still unclear. CDT has been shown to have am immune-modulatory effect, and it is possible that the absence of a functional holotoxin in an organism renders it more susceptible to the host immune response. The initiation of clearance of the CDT negative mutant also seems to correspond to the peak in the IgG response. It is possible that the elevated levels of IgG that are maintained after the two-month time point aid in the clearance of the organism.

The collected data indicates that *H. hepaticus* CDT plays a significant role in the development of inflammatory bowel disease by infected immune-altered mice. Additionally, the presence of a functional CDT holotoxin affects colonization, and is necessary for long-term persistence *H. hepaticus* in immune altered mice.

Mice infected with the CDT (-) mutant developed less severe disease than mutants infected with the wild-type strain of *H. hepaticus*. There was a drastic quantitative and qualitative difference in the nature of the typhlocolitis. None of the mice infected with

the CDT negative mutants developed significant submucosal inflammation, unlike the CDT positive strains.

Virulence factors, by definition, offer bacteria a distinct advantage in survival or pathogenesis. It is likely that CDT exerts its cytotoxic effects on cells that are normally undergoing continuous replication. These cells may include epithelial cell that line the intestine, as well as immune cells. Due to the nature of the gut, intestinal epithelial cells are constantly undergoing renewal and developmental maturation. This process is initiated by the stem cells of the crypt, and proceeds throughout the entirety of the villus, where they are eventually sloughed off at the tip of the villi. This would not provide a suitable home for bacteria that require attachment to host cells in order to colonize as these organisms would be sloughed off readily. By inducing cell cycle arrest, pathogenic organisms such as *C. jejuni* or *E. coli* could produce CDT in order to facilitate intestinal colonization. By inducing cell cycle arrest, an increase in the number of cells that would be conducive to their attachment could occur. It is also probable that CDT interferes with the developmental maturation of B cells and T cells. This would effectively dampen the immune response and lead to increased survival of the pathogen (18).

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