Helicobacter hepaticus increases intestinal injury in a rat model of necrotizing enterocolitis

Katerina Dvorak,1 Christine F. Coursodon-Boyiddle,2 Chelsea L. Snarrenberg,2 Anchasa Kananurak,3 Mark A. Underwood,4 and Bohuslav Dvorak1,2
1Department of Cellular and Molecular Medicine, The University of Arizona, Tucson, Arizona; 2Department of Pediatrics and Steele Children’s Research Center, The University of Arizona, Tucson, Arizona; 3Department of Medical Microbiology and Immunology, University of California-Davis, Davis, California; and 4Department of Pediatrics, University of California-Davis, Sacramento, California

Submitted 18 December 2012; accepted in final form 8 August 2013

We hypothesized that exposure of the developing intestinal mucosa to H. hepaticus infection will exacerbate NEC injury, decades of research and extensive clinical experience, its pathogenesis remains unclear. Recent studies suggest that bacterial colonization of newborn intestines plays a critical role in the disease onset. NEC may represent a final pathway for a heterogeneous group of intestinal insults (44), and for this reason it may be impossible to determine the origin and pathogenesis of this disease based purely on clinical observations. During the last two decades, experimental models of NEC have greatly advanced our knowledge of this disease and remain the key scientific tool for the development of novel therapies (17).

As addressed in more detail below, the premature neonate shows alterations in integrity of the epithelial layer, ability to maintain epithelial homeostasis, and formation of the mucus layer. These factors and alterations in regulation of inflammatory responses, including those due to bacteria:Toll-like receptor (TLRs) interactions, are all implicated in the pathogenesis of NEC (24). Evidence from animal models and human NEC tissue studies suggests that induction of TLR4 occurs early in NEC pathogenesis (3, 18, 25, 29-31), inducing expression of proinflammatory cytokines interleukin (IL)-6, IL-12, IL-18, IL-23, CXCL1, and tumor necrosis factor-α (TNF-α) (32, 40) (7). In models of H. hepaticus-induced colitis, TLR4 signaling plays an important role (37). However, the effect of H. hepaticus on the incidence of NEC, TLR4, and cytokine expression in the rat NEC model is not known.

Autophagy is a fundamental process that controls the catabolism of intracellular constituents (9) and has a crucial role in the maintenance of intestinal homeostasis (36). Uncontrolled autophagy leads to increased survival of intracellular bacteria and an uncontrolled proinflammatory response and has been implicated in the pathogenesis of Crohn’s disease (48). Our laboratory has discovered the induction of autophagy in the intestinal epithelium of NEC patients and in the rat NEC model (38). Recently, our initial observation was confirmed in the rat and mouse NEC models (43, 60).

The mucus coat is another line of defense protecting the epithelial surface against damage and pathogen invasion. The mucus layer is formed by mucins (Muc) and trefoil factors (Tff). Impaired production of Muc2 and Tff3 has been shown during NEC pathogenesis (5, 26). H. pylori thrives in the mucus layer of the stomach and alters mucin production and mucus clearance to create a more favorable niche for itself (42), but the effect of H. hepaticus on mucus secretion in the small intestine is not known.

We hypothesized that exposure of the developing intestinal mucosa to H. hepaticus infection will exacerbate NEC injury,
increase expression of TLR4 and proinflammatory cytokines, induce autophagy, and decrease production of intestinal Muc at the site of intestinal injury, the terminal ileum.

MATERIALS AND METHODS

Bacteria and animal model of NEC. This protocol was approved by the Animal Care and Use Committee of the University of Arizona (A-324801–95081). H. hepaticus (strain MU 94–1) (10) was obtained from the University of Missouri Research Animal Diagnostics Laboratory (Columbia, MO) by Dr. D. G. Besselsen (Director of University Animal Care, University of Arizona, Tucson, AZ). H. hepaticus isolate was grown in Brucella broth at 37°C with shaking under microaerophilic conditions (90% N₂-5% H₂-5% CO₂) for 1–3 days (10).

Pregnant Sprague-Dawley rats (Harlan Laboratories, Madison, WI) and their offspring were kept either in H. hepaticus-free conditions or colonized with H. hepaticus by being reared in an environment intentionally infected with the H. hepaticus pathogen. The presence of H. hepaticus in the intestinal tract was identified and confirmed by polymerase chain reaction (PCR) analysis (Fig. 1). Forty-one H. hepaticus-free pups (FF; n = 41) and 39 H. hepaticus-infected pups (FF+H.hep; n = 39) were used in this study. Neonatal rats were collected by caesarian section 24 h before their scheduled birth, and the first feeding started 2 h after delivery. Animals were hand fed five times daily with a total volume of 850 ml of rat milk formula per day at 4–6 μm, and stained with hematoxylin and eosin for histological evaluation of NEC. Pathological changes in intestinal architecture were evaluated using our previously published NEC scoring system (12, 26, 46). Histological changes in the ileum were scored by a blinded evaluator and graded as follows: 0 (normal), no damage; 1 (mild), slight submucosal and/or lamina propria separation; 2 (moderate), moderate separation of submucosa and/or lamina propria, and/or edema in submucosal and muscular layers; 3 (severe), severe separation of submucosa and/or lamina propria, and/or severe edema in submucosa and muscular layers, region villous sloughing; and 4 (necrosis), loss of villi and necrosis. Intermediate scores of 0.5, 1.5, 2.5, and 3.5 were also used to more accurately assess levels of ileal damage when necessary (12, 21). To determine the incidence of NEC, only animals with histological scores of 2 or greater were considered to have developed experimental NEC (26, 46).

RNA preparation and real-time PCR. Total RNA was isolated from ileal tissue (snap-frozen in liquid N₂) using the RNeasy Plus Mini Kit (Qiagen, Santa Clarita, CA) as described in the manufacturer’s protocol. RNA concentration was quantified by ultraviolet spectrophotometry at 260 nm, and the purity and integrity were determined using a NanoDrop (Thermo Fisher Scientific, Wilmington, DE) (27).

RT real-time PCR assays were performed to quantify steady-state mRNA levels of Muc2, Tff3 and selected cytokines (CXCL1, IL-1β, IL-6, IL-12, IL-17, IL-23, IFN-γ, and TNF-α), and TLRs (TLR2, TLR4, and TLR5). cDNA was synthesized from 0.2 μg of total RNA. Real-time PCR amplification was performed using Primer Express Software (Applied Biosystems). Target probe was labeled with fluorescent reporter dye. The following sequences were used: TNF-α (GenBank X66539): sense primer 5'-gtgatcggtcccaacaagga-3' , anti-sense primer 5'-gggccatggaactgatgaga-3' , and probe 5'-aacaaagtgggtcccc-3' ; Muc2 (GenBank BC036170): sense primer 5'-actgggaatgt-3' , and probe 5'-actgggaatgt-3'; Tff3, H. hepaticus-specific (p25F and p25R) (15), and TNF-α (used in the current study, lanes 3, 6, 9, and 12). The rat milk formula used for feeding of neonatal rats was prepared primarily from evaporated milk (13), and composition is described in Table 1.

Table 1. Preparation of rat milk formula

<table>
<thead>
<tr>
<th>Components</th>
<th>ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaporated milk;*</td>
<td>695</td>
</tr>
<tr>
<td>Bovine serum albumin;*</td>
<td>36</td>
</tr>
<tr>
<td>Intralipid 20% i.v. fat emulsion;*</td>
<td>292</td>
</tr>
<tr>
<td>Almond oil, ml</td>
<td>10</td>
</tr>
<tr>
<td>Teklad vitamin mixture CA 40060;*</td>
<td>4</td>
</tr>
<tr>
<td>Supplemental vitamin mixture;*</td>
<td>0.56</td>
</tr>
<tr>
<td>Salt mixture solution;*</td>
<td>10</td>
</tr>
<tr>
<td>Noncalcium mineral mixture;*</td>
<td>6</td>
</tr>
<tr>
<td>Calcium lactate solution (40 g/l);*</td>
<td>10</td>
</tr>
<tr>
<td>CaCO₃ solution (40 g/l);*</td>
<td>10</td>
</tr>
<tr>
<td>CaSO₄ solution (30 g/l);*</td>
<td>0.57</td>
</tr>
<tr>
<td>ZnSO₄ solution (380 g/l);*</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*A Carnation (Nestle, Solon, OH); *B Sigma-Aldrich, St. Louis, MO; *C Baxter, Deerfield, IL; *D Harlan Laboratories, Madison, WI; *E 16.7 g/kg riboflavin, 26.0 g/kg niacin, 13.9 g/kg pyridoxal, 929.4 g/kg inositol, and 14.0 g/kg ascorbic acid sodium salt; *F 257 g/l NaCl and 7 g/l KCl in distilled water; *G 842 μg/kg Na₃HPO₄, 152 g/kg MgSO₄, 4 g/kg FeSO₄· 7H₂O, 0.29 g/kg KI, 0.246 g/kg NaF, 0.156 g/kg Al₂SO₄, and 0.042 g/kg MnSO₄.
in 5% BSA to prevent nonspecific staining and incubated with goat anti-TLR4 polyclonal antibody (sc-30002; Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with Alexa Fluor 594-conjugated goat anti-secondary antibody (Molecular Probes, Eugene, OR) for 1 h and mounted with Vectashield Hard Set Mounting Medium containing YOYO-1 as a nuclear counterstain (Vector Laboratories). Negative control sections were treated with the same procedure in the absence of primary antibody; no immunostaining was observed in the negative controls (data not shown). Sections from each experimental group were immunostained for a specific antigen at the same time. Confocal laser scanning microscope (Zeiss LSM 510 NLO/META) equipped with a ×40 oil immersion objective was used to evaluate TLR4 staining.

Western blot analysis. Individual frozen ileal samples were homogenized with a hand-held homogenizer (Pellet Pestle; Kimble/Kontes, Vineland, NJ) in a 5× volume of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.1% SDS; 1% sodium deoxycholic acid; 1% Triton X-100; 50 mM DTT; 50 μg/ml aprotinin; 50 μg/ml leupeptin; and 5 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was collected.

Total protein concentration was quantitated using the Bradford protein assay. For protein analysis, 40 μg of protein were added to an equal volume of 2× Laemmli sample buffer and boiled for 5 min. The samples were run on 10% polyacrylamide gels at 110 V for 1.5 h. Protein was transferred to Immuno-Blot PVDF membranes (Bio-Rad) at 100 mA for 1.5 h. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (Sigma) at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG (Santa Cruz Biotechnology). Proteins were visualized by chemiluminescent system (Pierce, Rockford, IL) and exposed to X-ray film.

Enumeration of Muc2- and Tff3-positive cells. Serial sections of the ileum were stained for either Muc2 or Tff3. Briefly, after deparaffinization and rehydration, sections were incubated with either rabbit anti-Muc2 polyclonal antibody (Santa Cruz Biotechnology) or rabbit polyclonal Tff3 antibody (54) for 30 min, washed with PBS three times, and incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) for 30 min. Vectastain Elite ABC reagent (Vector Laboratories) was then applied, followed by diaminobenzidine as a substrate. Sections were counterstained with hematoxylin, dehydrated, and mounted on cover slips. Muc2- and Tff3-positive cells were counted from nine animals per experimental group, and the total number of epithelial cells per crypt-villus unit was also enumerated. Fifteen crypt-villus units were counted for each animal.

Statistical evaluation. All numerical data are expressed as means ± SE. Statistical analyses between FF and FF+H. hepaticus groups were performed by the Student’s t-test at the 95% confidence level. Analysis of NEC score between groups was accomplished using the Kruskal-Wallis test for nonparametric values followed by pairwise comparison using the Mann-Whitney test. The Pearson’s χ² test was used to analyze differences in incidence of NEC between groups. All statistical analyses were conducted using the statistical program StatPlus:mac LE for Macintosh computers (AnalystSoft).

RESULTS

Exposure to H. hepaticus infection exacerbates severity and incidence of NEC. The Helicobacter strain used in this study was confirmed by PCR technique as a strain of H. hepaticus (Fig. 1) (15). There was no statistically significant difference in the survival of caesarian section-delivered premature rats between experimental groups [FF+H. hep 90% (35/39) and FF 93% (38/41)]. Damage to the villi and submucosa in the ileum was significantly higher in the FF+H. hep group with a mean value of NEC injury of 2.01 ± 0.09 compared with 1.68 ± 0.10 in the FF group (P ≤ 0.01; Fig. 2A). The incidence of NEC was higher in the FF+H. hep group [71% (25/35)] than the FF group [39% (15/38); P ≤ 0.01; Fig. 2B].

H. hepaticus increases intestinal expression of TLR4 in a rat model of NEC. Gene expression of TLR4 in the ileum was increased two- to threefold in the FF+H. hep group compared with the FF group (P ≤ 0.02; Fig. 3A). There were no statistically significant differences in TLR2 and TLR5 mRNA levels between these two groups (results not shown). Western blot analysis showed two- to threefold higher levels of TLR4 protein in the ileum of the FF+H. hep group compared with the FF group (P ≤ 0.01; Fig. 3B). Protein levels of TLR2 were similar in both groups (results not shown). Confocal microscopy revealed that TLR4 signal was localized predominantly in the cells of the lamina propria in the ileum from the FF+H. hep group (Fig. 3C) with significantly fewer TLR4-positive cells observed in the ileum of FF rats (P ≤ 0.01).

H. hepaticus increases inflammatory response in the ileum. Gene expression of CXCL1, IL-1β, IL-6, IL-12, IL-17, IL-23, and IL-25 was significantly increased in the ileum of FF+H. hep groups compared with the FF group (P ≤ 0.02; Fig. 4A). The expression of TLR4 in the ileum was increased two- to threefold in the FF+H. hep group compared with the FF group (Fig. 4B). The incidence of NEC was higher in the FF+H. hep group [71% (25/35)] than the FF group [39% (15/38); P ≤ 0.01; Fig. 2B].

Fig. 2. Severity and incidence of necrotizing enterocolitis (NEC) in neonatal rat model. A: histological NEC score in the H. hepaticus-free formula-fed rats (FF) (Δ; n = 38) and in the H. hepaticus-infected formula-fed rats (FF+H. hep) (●; n = 35). Ileal damage was assessed using the histological scoring scale of 0 (normal) to 4 (necrosis), as previously described (7, 12, 26). B: incidence of NEC in the neonatal rat model of NEC. Animals with scores ≥2 are considered NEC positive, and animals with ileal damage <2 do not have NEC. *P ≤ 0.01 vs. FF, χ² analysis.
IFN-γ and TNF-α was evaluated in the terminal ileum of studied animals. Exposure to H. hepaticus increased IL-23 mRNA levels in the ileum ~20 times compared with the FF group (P < 0.05; Fig. 4A). Western blot analysis showed IL-23 protein levels about three to four times higher in the FF + H.hep group compared with the FF group (P < 0.01; Fig. 4B).

Expression of proinflammatory IL-1β, CXCL1, and IL-12 was significantly increased in rats exposed to H. hepaticus compared with the FF group (Fig. 5). There were no statistically significant differences in IL-6, TNF-α, and IFN-γ mRNA levels between these two groups (results not shown). Gene expression of IL-17 was not detected in the ileum of these animals (results not shown).

**Evaluation of Muc2 and Tff3 in the ileum.** Ileal Muc2 and Tff3 gene expression and production were evaluated using real-time PCR and immunohistochemistry (Table 2). Gene expression for both Muc2 and Tff3 was similar in all experimental groups. Enumeration of Muc2- and Tff3-positive cells in the terminal ileum showed no differences between these two groups. These results indicate that exposure to H. hepaticus does not affect the formation of the mucus layer in this model.

**H. hepaticus induces autophagy in the ileum of a rat model of NEC.** Induction of autophagy is frequently evaluated by following the phospholipid conjugation of LC3I (cytosolic form) to LC3II (membrane-bound form) (47). Detection of LC3II serves as an essential autophagosomal marker, and the ratio between these two LC3 proteins (LC3II/LC3I) correlates with the number of autophagosomes (58). To determine the effect of H. hepaticus on autophagy, LC3 protein levels were evaluated in the ileum of rat pups. In the FF + H.hep group, the expression of the LC3II isoform was markedly higher compared with FF pups (Fig. 6A). The LC3II-to-LC3I ratio in FF + H.hep rats was increased significantly, suggesting the activation of autophagy compared with FF rats (n = 9; P < 0.02; Fig. 6A).

The p62 protein recognizes toxic cellular waste, which is then scavenged from the cell by autophagy. Direct interaction of p62 with LC3 complex leads to the degradation of p62 by the autophagy-lysosome system. Thus, degradation of p62 protein serves as a unique marker of autophagy activation. Protein levels of p62 in the ileum were markedly decreased in the FF + H.hep group compared with rats from the FF group (Fig. 6B), suggesting increased autophagic degradation in the autolysosome (consistent with the changes in the LC3II-to-LC3I ratio).

**DISCUSSION**

Although most long-term outcomes for prematurely born babies are gradually improving, NEC remains a significant issue.
clincial problem. The exact cause of NEC is not known, but critical elements in disease development include immaturity of virtually every aspect of intestinal innate immunity, enteral feeding, and inappropriate gut colonization (dysbiosis) (6, 44). Decreased microbial diversity, alteration in the composition of the intestinal microbiota, and colonization with unfavorable bacteria are frequently observed in feces from NEC patients (57). Indeed, the primary sites of NEC injury (the terminal ileum, ileocecal area, and ascending colon) are niches associated with high numbers and diversity of intestinal microbes. However, a specific pathogen predisposing neonates to develop NEC has not been identified (53).

_H. hepaticus_ is a Gram-negative bacterium of the phylum Proteobacteria, which causes chronic hepatitis, hepatocellular carcinoma, typhlocolitis, and colorectal cancer in murine models (55). Although _H. hepaticus_ has not been found in humans, other EHS (_H. bilis, H. fennelliae_, and _H. pullorum_) have been isolated from humans with intestinal diseases such as gallbladder cancer, cholecystitis, pancreatitis, and chronic diarrhea (16, 22, 52). In this study, for the first time, we demonstrate that infection with _H. hepaticus_ increases the incidence of NEC and exacerbates intestinal injury in the rat NEC model.

These observations prompt two compelling questions. First, given that _H. hepaticus_ infection is common and often not clinically evident in laboratory rodents (as high as 59% infection rate) (55), could some of the conflicting or ambiguous results reported from rodent models of NEC be related to undiagnosed _H. hepaticus_ infection? At the very least, these data suggest the importance of regular testing for _H. hepaticus_ in future experimental NEC studies (4). Second, given that _Helicobacter_ species are widespread in the human population (34), could colonization or infection with EHS be contributory to increased susceptibility of premature infants to develop NEC?

In premature babies, NEC typically develops between 2 and 8 wk after delivery, which is similar to the timeline necessary for gut colonization (44) and appears to be associated with dysbiosis. Human studies showed an increase in fecal _Proteobacteria_ in NEC, but predominantly from the family Enterobacteriaceae (33). Recent studies from the feces of premature infants with and without NEC did not identify organisms of the family _Helicobacteriaceae_ (45, 53). While stool specimens may not directly reflect the bacterial population in the distal ileum or proximal colon, it seems unlikely that EHS directly...

---

**Table 2. Effect of _H. hepaticus_ on Muc2 and Tff3 expression in the ileum**

<table>
<thead>
<tr>
<th>Group</th>
<th>Muc2 mRNA</th>
<th>Muc2-Positive Cells</th>
<th>Tff3 mRNA</th>
<th>Tff3-Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF</td>
<td>1.00 ± 0.49</td>
<td>9.5 ± 0.5</td>
<td>1.02 ± 0.28</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>FF + H.hep</td>
<td>0.96 ± 0.29</td>
<td>9.9 ± 0.3</td>
<td>0.82 ± 0.12</td>
<td>5.1 ± 0.5</td>
</tr>
</tbody>
</table>

Values for Muc2 and Tff3 mRNA are means ± SE; _n_ = 8–10 animals/experimental group. Nos. for Muc2-positive cells and Tff3-positive cells are expressed as mean Muc2- or Tff3-positive cells/100 epithelial cells ± SE. Muc2, mucus; Tff, trefoil factor; FF, _Helicobacter hepaticus_-free condition; FF + H.hep, _H. hepaticus_ infected. The mean steady-state mRNA level for the FF group was assigned a value of 1.00, and mean mRNA level from the FF + H.hep group was determined relative to this number.
Fig. 6. Effect of *H. hepaticus* on expression of LC3II and p62 proteins. A: left, representative LC3 bands from Western blot analyses are shown for the FF and FF+*H. hep* groups. Conjugation from LC3I (17 kDa) into the LC3II (15 kDa), an autophagosomal membrane-incorporated isoform, is observed in the FF+*H. hep* group. Right, densitometry quantification of Western blot analysis for LC3 presented as a ratio of LC3II/LC3I (n = 7; *P* ≤ 0.01 vs. FF). B: p62 mRNA levels evaluated using real-time PCR. The mean steady-state mRNA level for the FF group was assigned a value of 1.0, and mean mRNA level for the FF+*H. hep* group was determined relative to this number. Values are means ± SE; n = 14–16 animals/experimental group. *P* = 0.02 vs. FF. C: left, representative p62 (62-kDa) bands from Western blot analyses are shown for the FF and FF+*H. hep* groups. Right, densitometric values were normalized to β-actin and to FF value (n = 7; *P* ≤ 0.01 vs. FF).

trigger NEC in the neonate. Rather, it is more likely that the proinflammatory effects of *H. hepaticus* in the rat are analogous to the proinflammatory effects of *Enterobacteriaceae* in the neonate. *Enterobacteriaceae* can both trigger a proinflammatory response and outcompete commensal microbes in a proinflammatory environment.

*H. hepaticus* induces experimental colitis in adult immunocompromised mice with a fully developed gastrointestinal tract (22). However, the effect of *H. hepaticus* on the developing intestine has not been studied. In our initial experiments, we exposed normal newborn rats nursed by a surrogate dam to *H. hepaticus* infection during the 1st wk of life. There were no pathological changes observed in the small intestine of these neonatal rats (results not shown). Subsequently, we tested the effect of *H. hepaticus* on the developing gut in a well-established rat model of NEC, where the immature intestine is challenged with formula feeding and asphyxia followed by cold stress to induce NEC injury. The activation of TLR4 is reported in the intestines of NEC patients and in the experimental NEC models (18, 25, 29–31). Moreover, NEC injury is reduced in TLR4 mutant mice (25, 30) and in the enterocyte-specific TLR4 knockout mice (49). Our data clearly indicate that *H. hepaticus* infection stimulates TLR4 but does not affect TRL2 expression. These observations are consistent with previous reports of increased TLR4 signaling in models of *H. hepaticus*-induced colitis (37, 52).

In NEC models, TLR4 signaling induces immune response and secretion of proinflammatory cytokines (8, 29, 31). The small intestine is the largest immune organ in the body, and its activation is critical in the protection against enteric pathogens. In *H. hepaticus*-induced NEC, the pattern of cytokine induction differed somewhat from reports of standard NEC with marked increases in IL-1β, CXCL1, IL-12, and IL-23 but no significant alteration in IL-6, IFN-γ, or TNF-α, which are commonly increased in the rat NEC model (7, 11, 14, 20, 39). It is challenging to compare the current data with previous reports given the unknown colonization rates of *H. hepaticus* in previously reported studies.

TLR4 signaling induces IL-23, which is secreted by activated dendritic cells and macrophages and triggers intestinal inflammation and host defense against microbial pathogens (41). IL-23 is an essential driving force for inflammatory response (35), and the depletion of IL-23 prevents the development of *H. hepaticus*-induced colitis (16, 28, 41). IL-23 amplifies release of proinflammatory cytokines (such as IL-1β, IL-6, and TNF-α), but can also regulate production of Th17 cytokines, especially IL-17 (16). Interestingly, the presence of IL-17 mRNA was not detected in any of studied samples, suggesting that the Th17 response may be delayed in the immature intestine of neonatal rats.

Previously, we reported the activation of autophagy in the intestinal epithelium from babies with NEC and in the rat NEC model (38). In addition, we showed that the inhibition of autophagy reduces experimental NEC (38). Recently, Neal et al. and Yu et al. extended our observation by identifying that increased autophagy is a cause (not a consequence) of NEC.
and requires TLR4 activation (43, 60). Results from the present study are in agreement with these reports (43, 60) and further support our original hypothesis that inappropriate activation of autophagy exacerbates NEC (38). At a basal level, autophagy acts as a protective mechanism (19), but exaggerated autophagy increases mucosal injury. Because TLR4 signaling not only induces autophagy (43) but also activates the proinflammatory response (23), we speculate that H. hepaticus infection increases NEC via both mechanisms.

We did not observe any alteration in production of Muc2 or TFF3 in response to H. hepaticus infection, suggesting that changes in these contributors to the mucin layer are not part of the mechanism by which H. hepaticus induces NEC and that H. hepaticus infection in the ileum differs from H. pylori infection in the stomach in this regard.

In summary, this study demonstrates that H. hepaticus infection increases the incidence and severity of experimental NEC through several mechanisms involving increased TLR-4 signaling. Future rodent studies of NEC should include testing for infection with H. hepaticus. Whether maternal and/or neonatal exposure to Helicobacter infection may increase the risk of NEC in premature babies is a challenging question given the difficulty of sampling the microbiota of the distal ileum and proximal colon. Helicobacter infections are associated with TH17 and TH1 responses. Analysis of Helicobacter-associated cytokines (e.g., IL-17, IL-23, IFN-γ, and IL-12) in the serum of infants with and without NEC may be valuable in addressing this question. Studies of the microbiota and metagenome of the distal ileum and proximal colon from samples obtained at the time of surgical resection or at autopsy from infants with NEC and other non-NEC intestinal diseases (e.g., spontaneous ileal perforation) would be more definitive.

ACKNOWLEDGMENTS


GRANTS

This work was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development Grant HD-039657 (to B. Dvorak) and a gift from People Acting Now Discover Answers.

DISCLOSURES

B. Dvorak has grants with Mead Johnson, Meiji Dairies Co., and NIH. There are no conflicts of interest.

AUTHOR CONTRIBUTIONS


REFERENCES


