The role of luminal factors in the recovery of gastric function and behavioral changes after chronic *Helicobacter pylori* infection

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Chronic *Helicobacter pylori* infection is the hallmark of *H. pylori* infection (7, 13). We have shown that, in mice, *H. pylori* infection induces functional and morphological changes in the gastric and spinal neural circuitry that are progressive and lymphocyte dependent (2). Some of these changes persist after bacterial eradication, suggesting that postinfective changes and immune activation are long lasting. In particular, altered feeding patterns, reminiscent of early satiety, are present up to 2 mo posteradication (posteradication) (3).

Persistence of symptoms may relate to luminal factors, which maintain low-grade inflammation after *H. pylori* eradication. Gut barrier function is crucial in limiting the effect of luminal antigens on the immune system. Thus, in this study, we examined whether the antigenic or bacterial content of the gut influences the changes in gastric function and feeding behavior induced by the *H. pylori* chronic infection. Specifically, we determined whether exposure to *H. pylori* antigen or probiotic bacteria influences host physiology.

Our results suggest that the bacterial content of the gut, as well as the presence of relevant antigens, influences the rate of recovery of host pathophysiology induced by *H. pylori* infection.

MATERIALS AND METHODS

**Animals.** Male BALB/c mice (Harlan, Indianapolis, IN) were purchased at the age of 6–8 wk and housed in a conventional specific pathogen-free unit at McMaster University Central Animal Facility. All experiments were conducted with approval from the McMaster University Animal Care Committee.

**Chronic *H. pylori* infection.** Mice chronically infected with *H. pylori* Sydney strain for 4 mo (*n* = 64) and a group of uninfected controls were used (*n* = 27). Additional mice infected with *H. pylori* were used to monitor the establishment of a chronic infection (*n* = 14). Every 2 wk, beginning at 2 wk postinfection, 2 mice per group were euthanized, and *H. pylori* infection was verified using Warthin-Starry staining. Gastric emptying and 24-h feeding patterns were assessed at 4 mo of chronic infection. Additional mice were euthanized and used for ex vivo intestinal permeability measurements. *H. pylori* eradication therapy was administered thereafter using antibiotic-containing food pellets (Bio-Serv, Frenchtown, NJ) for 2 wk. Gastric emptying was reassessed 2 wk and 2 mo posteradication. Feeding patterns and intestinal permeability were reassessed 2 mo posteradication.

**Inflammation.** Stomach samples (Swiss rolls) were preserved in 10% formalin and then stained with hematoxylin and eosin (H & E).

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H & E-stained and Warthin-Starry-stained slides were examined under light microscopy to confirm *H. pylori* eradication after antimicrobial therapy and assess gastric inflammation. Mononuclear cell (MN) scores were graded in the corpus on a scale of 0–3 as described previously (2).

Immunostaining for CD3+ cells was performed on stomach paraffin sections using a modified method described previously (2). Rabbit anti-mouse CD3 (1:300; Dako, Glostrup, Denmark) was used as primary antibody followed by biotinylated swine anti-rabbit (1:300, Dako) and streptavidin peroxidase conjugate (1:600, Dako). The antibodies were visualized using 3-aminio-9-ethylcarbazole and counterstaining with Mayers hematoxilyn. Negative controls were performed in the absence of primary antibody. CD3+ cells were counted in two slides per mouse (*n* = 12/group) and averaged. CD3+ cells present in three randomly selected fields in the corpus and antrum separately (x63, mucosa and submucosa) were counted. Samples from the jejunal loop were obtained at the end of each permeability experiment to test for tissue viability. Samples were fixed in 10% formalin, stained with H & E, and examined for tissue damage as a result of hypoxia using light microscopy. Gross villous architecture and the presence of cell desquamation and edema at villi tips were investigated.

**H. pylori antibody measurement.** Levels of anti-*H. pylori* IgG1 and IgG2A were measured at 2 mo post-eradication by ELISA using biotinylated goat anti-mouse IgG2a and IgG1 (Southern Biotechnology Associates, Birmingham, AL) as described previously (9).

**Gastric emptying.** Mice were gavaged with 0.2 ml of 40% barium water or 100 μl of 106 Listeria monocytogenes (L. monocytogenes) R0011 and *L. helveticus* R0052 (Lacidofil) for 2 wk immediately after eradication therapy. Uninfected mice received maltodextrin treatment on a daily basis for 2 wk. Gastric emptying was reassessed at the end of probiotic treatment (2 wk post-eradication) and at 2 mo post-eradication. Stomach samples were obtained at sacrifice and fixed in formalin for Warthin-Starry stain and for CD3+ cell counts.

**Probiotic treatment post-eradication.** Groups of mice received by daily gavage either 100 μl of placebo-maltodextrin dissolved in sterile water or 100 μl of 106 Lactobacillus rhamnosus (L. rhamnosus) R0011 and *L. helveticus* R0052 (Lacidofil) for 2 wk immediately after eradication therapy. *H. pylori* antigen was prepared from fresh *H. pylori* cultures using liquid brain-heart infusion-based media as described previously (2). The bacteria were then gently centrifuged, and pellets were resuspended in saline and homogenized/ disrupted with a sonicator. The concentration of bacterial antigen was adjusted with saline (100 μl/mouse). Gastric emptying was reassessed at the end of *H. pylori* antigen administration (2 mo post-eradication). Stomach samples were obtained at sacrifice and fixed in formalin for Warthin-Starry stain and for CD3+ cell counts.

**Detection of L. rhamnosus R0011 and L. helveticus R0052 in feces.** Fresh fecal pellets were collected aseptically from the anal region into a sterile cryogenic tube containing 0.9% saline and 10% glycerol while the animals were kept in Plexiglas restrainers. For analysis, 100 μl of fecal solution were pipetted into 25 ml of de Man, Rogosa, and Sharpe broth plus antibiotics (5.9 mg/ml phosphomycin and 18.6 mg/ml sulfamethoxazole) and 1 mg/ml in HSO4 0.02 M trimethoprim. Vancomycin (5 mg) was added for selective *L. rhamnosus* R0011 and ciprofloxacin (5 mg) for *L. helveticus* R0052 growth and was cultured anaerobically at 37°C for 48 h.

For DNA extraction and PCR amplification, 1.5 ml of each culture solution were centrifuged, and bacteria pellet was collected. Bacterial genomic DNA was extracted by Wizard Genomic DNA purification kit (Promega, Madison, WI) according to manufacturer’s instructions. PCR amplification was performed in a DNA Thermal Cycler 480 (Perkin Elmer) as follows: in a 0.5 ml PCR tube, 1 μl (0.2 μg) of DNA was added to 49 μl of PCR reaction mixture containing 200 μM of each dNTP, 1.5 mM MgCl2, 10 pmol amounts of each primer and 2.5 U of Taq DNA. PCR cycling parameters were 3 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C; after the last cycle, we added a final extension 7 min at 72°C. PCR product was visualized by ethidium bromide staining in 2% agarose gels. R0011 was used as a positive control, and *L. casei* R0215 was used as negative controls.

PCR primer pairs were as follows: *L. helveticus* R0052: sense 5’-ATTTTGCAACT GTTACTCCATC-3’, antisense 5’-GCATAAT-AGTCTTGACTACG-3’; *L. rhamnosus* R0011 (two pairs of primer sets): sense 5’-GACAAACTGTATTTCCCAC-3’, antisense 5’-TC-CAATGTTCCTAAACGAG-3’ and sense TCAGTAGACACCTAC-CGG-3’, antisense 5’-GTGTTAAAAGCTTGGACCGG. The first pair of primers can amplify *L. zeae* ATCC 393 and its prophase (phiAT3) due to the high homology between this phage and R0011.
prophage, LarhR11-1. Therefore, only samples that were positive for both primer sets were considered positive for R0011.

**Statistical analysis.** Data are presented as means ± SD or medians with interquartile ranges when appropriate. Data was analyzed using either two-way ANOVA, Kolmogorov-Smirnov test or non-paired t-test as appropriate. The spearman rank correlation test was used to test the strength of association between parameters. A *P* value of <0.05 was considered statistically significant.

**RESULTS**

**Effect of chronic *H. pylori* infection on inflammation.** Chronic *H. pylori* infection (4 mo postinfection) induced chronic active inflammation, located mainly in the submucosal layer of the proximal stomach. The mononuclear cell score in the corpus was 0.6 ± 0.6 and 2.0 ± 0.7 (*P* < 0.01) in uninfected controls and *H. pylori*-infected mice, respectively. CD3⁺ cell scores in controls were 3.8 ± 3.0 and 0.8 ± 0.9 in corpus and antrum, respectively. During *H. pylori* infection, they increased to 14.3 ± 7.1 and 4.5 ± 1.2 (both *P* < 0.01 vs. uninfected controls).

**Effect of chronic *H. pylori* infection on gastric emptying.** In accordance with previous results (3), the percentage of retained barium during *H. pylori* infection was 30% higher than in uninfected mice (Fig. 1, before eradication). Gastric emptying fully normalized at 2 mo posteradication (Fig. 1, 2 mo after eradication).

**Effect of chronic *H. pylori* infection on body weight.** In accordance with previous results (2, 3), there were no differences in body weight between uninfected and chronically *H. pylori*-infected mice (24.3 ± 2.6 g and 24.7 ± 3.0 g, respectively).

*H. pylori* antigen delays recovery of inflammation and gut function after bacterial eradication. In mice previously infected with *H. pylori*, administration of crude *H. pylori* antigen maintained delayed gastric emptying for up to 2 mo posteradication (Fig. 1, 2 mo posteradication). Delayed gastric emptying correlated with increased CD3⁺ cell counts (Spearman rank correlation test, *P* < 0.05). Antigen-treated mice had IgG1 and IgG2A values nine- and 23-fold higher, respectively, compared with placebo-treated mice. Antigen administration did not affect gastric emptying in uninfected mice.

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**Fig. 1.** A: gastric emptying was delayed in mice infected with *Helicobacter pylori* (*H. pylori*) (*n* = 36) compared with uninfected controls (*n* = 20). B: two weeks after eradication, gastric emptying improved in previously infected mice treated with placebo (*n* = 12, *P* = 0.02 vs. *H. pylori*) but remained delayed compared with uninfected controls (*P* = 0.04). Previously infected mice treated with probiotics (*n* = 12) improved their gastric emptying (**P* < 0.01 vs. *H. pylori*) and were similar to uninfected controls but were not different from placebo-treated mice. C: two months posteradication, gastric emptying normalized in placebo-treated (*n* = 12) mice but remained abnormal in antigen-treated mice (*n* = 12, *P* < 0.01 vs. uninfected antigen, **P* = 0.02 vs. uninfected placebo). D: degree of delayed gastric emptying was associated with the CD3⁺ cell counts in stomach (*r*², 0.6; *P* = 0.01).
Detection of L. rhamnosus R0011 and L. helveticus R0052 in feces. Figure 2 shows an example of positive detection for L. rhamnosus R0011 and L. helveticus R0052 in feces at the end of probiotic feeding. All Lacidofil-fed mice tested positive for the specific probiotics in feces at the end of the probiotic administration period. No cross contamination was observed in mice gavaged with placebo.

Probiotics improve markers of inflammation post eradication. In placebo-treated mice, there was persistent infiltration with CD3⁺ T cells in the submucosal layer of the corpus and antrum 2 mo after eradication of H. pylori. In contrast, previously infected mice treated with probiotics exhibited a 60% lower MN score in corpus compared with placebo-treated mice (2.2 ± 0.5 vs. 0.8 ± 0.4, P = 0.01). The CD3⁺ T cell infiltrate in both corpus and antrum was also reduced by probiotic therapy (Fig. 3). There was no overt inflammation in the jejunal segments of H. pylori-infected mice compared with uninfected controls.

Effect of probiotics on gastric emptying after H. pylori eradication. At 2 wk post eradication, previously H. pylori-infected mice treated with probiotics had returned to uninfected

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Fig. 2. Example of PCR detection of probiotics in feces. Two primers were used to detect R0011 (A and B) and one primer to detect R0052. Bands show negative detection of probiotics in feces of uninfected mice treated with maltodextrin (Uninf-pla), infected mice treated with maltodextrin placebo (Hp-Pla), and negative control R0215. Positive detection is shown in 2 infected mice treated with probiotics (Hp-Lacidofil-1 and -2), and in positive controls using R0011 and R0052.

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Fig. 3. A: at 2 mo post eradication, a persistent, predominantly submucosal chronic infiltrate was observed in the gastric body of mice previously infected with H. pylori compared with uninfected controls. Treatment with probiotics decreased chronic gastritis (photograph magnification ×10). B: CD3⁺ cell counts were increased both in corpus (P = 0.01) and antrum (P < 0.0001) of previously infected mice compared with uninfected controls. Treatment with probiotics normalized the CD3⁺ cell counts in both corpus and antrum (P = 0.04 and P < 0.001 vs. placebo-treated mice). Quantification performed in mucosa and submucosa; 2 slides/mouse (n = 12/group) (magnification ×63) and averaged.
gastric emptying values. However, there were no statistical differences between previously infected mice treated with placebo or probiotics 2 wk after eradication. Two months after eradication, previously infected mice that had been treated with probiotics tended to have faster gastric emptying than placebo-treated mice (Fig. 1).

Probiotics improve recovery of 24-h feeding behavior after H. pylori eradication. At 4 mo, frequency of eating bouts per 24 h was higher in H. pylori-infected mice compared with uninfected controls (Fig. 4). In placebo-treated mice, altered feeding patterns remained unchanged for at least 2 mo post-eradication. In contrast, previously infected mice treated with probiotics had a similar number of eating bouts per 24 h as uninfected time-point controls.

Effect of probiotics on paracellular small intestinal permeability. Only intestinal segments with intact morphology at the end of experiments were included in the study. Jejunal permeability to $^{51}$Cr-EDTA and $^{14}$C-mannitol was increased in H. pylori-infected mice compared with uninfected controls (Fig. 5). Bacterial eradication combined with probiotics tended to improve paracellular permeability to $^{51}$Cr-EDTA ($P = 0.35$ vs. uninfected controls), but this did not reach statistical significance vs. placebo-treated mice. Probiotics did not affect membrane permeability as assessed by $^{14}$C-mannitol.

**DISCUSSION**

The aim of our study was to investigate whether the antigenic or bacterial content of the gut influences the rate of recovery of host physiology induced by chronic H. pylori infection after bacterial eradication.

We have previously shown that altered gastric emptying improves at 2 wk post-eradication and completely normalizes 2 mo post-eradication (3). The probiotic combination L. rhamnosus R0011 and L. helveticus R0052 administered immediately after H. pylori eradication accelerated recovery of gastric chronic inflammation. In contrast, previously infected mice that received H. pylori antigen had persistent CD3$^+$ cell counts in the stomach that correlated with persistent delayed gastric emptying post-eradication.

In the chronic model of H. pylori infection, the degree of neural impairment is proportional to the extent of the chronic inflammatory infiltrate (2). In the present study, we have extended this observation and showed that the degree of delayed gastric emptying is proportional to the CD3$^+$ cell counts in the stomach. Furthermore, H. pylori antigen-treated mice had persistent delayed gastric emptying post-eradication compared with placebo-treated controls. This was accompanied by higher anti-H. pylori antibody titers, suggesting a
heightened immune response after luminal H. pylori antigen administration. In contrast, probiotic therapy significantly decreased the number of CD3+ cells in the stomach of previously infected mice compared with placebo-treated mice in parallel with a faster recovery of gastric emptying. Thus the effect of probiotics on gastric emptying recovery may be mediated through a faster recovery of the chronic inflammatory response to H. pylori.

H. pylori-infected mice ate more frequently but smaller amounts of food per feeding bout compared with uninfected controls. This resulted in a similar total amount of food consumed per 24 h. The pattern is reminiscent of that observed frequently in patients with functional dyspepsia who have difficulty consuming regular size meals and therefore snack frequently throughout the day. Administration of probiotics normalized postinfective altered feeding behavior. It is possible that chronic inflammation in the stomach alters ascending neural pathways, resulting in abnormal feeding behavior, and that probiotics improve this through an effect on H. pylori-associated gastritis. However, other mechanisms such as direct modulation of neuroendocrine pathways by probiotics cannot be ruled out.

It has been shown that H. pylori infection alters gastric permeability in vivo and also on epithelial cell lines (6, 14, 15). The underlying mechanisms may include impaired mucus-bicarbonate barrier, disruption of tight junctions (occludin, ZO), and increase in transepithelial permeability by H. pylori. These alterations may be long lasting and linked to chronic inflammation because bacterial eradication has been shown to improve gastric permeability only in those mice with significant improvement of chronic gastritis (14). A recent clinical study has suggested that intestinal permeability is altered in subjects with H. pylori infection (5). This may be more clinically relevant than changes in gastric permeability because the intestine represents a larger area of antigen and nutrient processing. The H. pylori-induced defect in intestinal barrier could result in chronic immune stimulation and bystander antigen stimulation even after H. pylori eradication. We measured permeability in ex vivo jejunal segments using a combination of two macromolecules to assess paracellular and membrane permeability. 51Cr-EDTA is an established marker for paracellular permeability, and C14-mannitol is considered to be a marker for membrane permeability in in vivo studies. Both permeability to 51Cr-EDTA and mannitol were increased in H. pylori-infected mice. Bacterial eradication did not normalize intestinal permeability. Treatment with probiotics tended to improve paracellular permeability, but this did not achieve statistical significance compared with those previously infected and treated with placebo. Probiotics did not modify membrane permeability. In contrast to the probiotic-induced normalization of feeding behavior, combined administration of L. rhamnosus R0011 and L. helveticus R0052 had a modest effect on increased intestinal permeability after bacterial eradication. Thus resolution of altered feeding behavior and gastric emptying abnormalities by probiotics do not seem to be mediated principally by an improvement in small intestinal permeability in the chronic model of H. pylori infection. This finding is in disagreement with an earlier study using a stress model in the rat that showed that this combination of probiotic bacteria could protect against bacterial translocation (24). However, it supports the findings in an acute model of H. pylori in which L. rhamnosus R0011 and L. helveticus R0052 reduced H. pylori-induced antrum inflammation but not apoptosis (10).

In conclusion, using a murine model of chronic H. pylori infection and postinfective gut dysfunction we have shown that administration of L. rhamnosus R0011 and L. helveticus R0052 after H. pylori eradication accelerates recovery of gastric motor function and normalizes altered feeding behavior. This is associated with improvement in chronic gastric inflammation by probiotics but not with full recovery of intestinal barrier abnormalities. Treatment with luminal antigen related to the triggering infectious agent maintains gastric dysfunction long after bacterial eradication. The results suggest that specific probiotics may be useful in improving the rate of symptomatic relief in patients with dyspepsia after H. pylori eradication.

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