Immune-Mediated Neural Dysfunction in a Murine Model of Chronic *Helicobacter pylori* Infection

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Background & Aims: **Neuromuscular changes producing dysmotility and hyperalgesia may underlie symptom generation in functional gastrointestinal disorders. We investigated whether chronic *Helicobacter pylori*–induced gastritis causes neuromuscular dysfunction.** ***Methods:*** **In vitro muscle contractility and acetylcholine release were evaluated in mice before and after *H. pylori* eradication. *H. pylori* colonization and gastritis were graded histologically. Substance P (SP)-, vasoactive intestinal polypeptide (VIP)-, and calcitonin gene-related peptide (CGRP) immunoreactivity (IR) and macrophages were studied by immunohistochemistry.** ***Results:*** **In Balb/c mice, chronic *H. pylori* infection did not affect muscle function but augmented antral relaxation after nerve electric field stimulation. Infected mice had lower acetylcholine release by electric field stimulation and had higher density of SP-, CGRP-, and VIP-IR nerves in the stomach and of SP- and CGRP-IR in the spinal cord. Cholinergic nerve dysfunction worsened progressively and was associated with increasing macrophage and mononuclear but not polymorphonuclear infiltrate or bacterial colonization. SCID mice had unchanged acetylcholine release despite high *H. pylori* colonization and macrophage infiltration. Eradication of *H. pylori* normalized functional and morphologic abnormalities except for increased density of gastric SP- and CGRP-IR nerves.** ***Conclusions:*** ***H. pylori* infection induces functional and morphologic changes in the gastric neural circuitry that are progressive and lymphocyte dependent, and some persist after *H. pylori* eradication. The data have direct implications regarding the role of *H. pylori* infection in functional dyspepsia.**

Functional gastrointestinal disorders such as functional dyspepsia or irritable bowel syndrome constitute the most common category of problems seen by gastroenterologists.[1] However, our ability to manage these patients effectively is constrained by a limited understanding of the underlying pathophysiology. The heterogeneity of these disorders is reflected not only in their clinical presentation but also in the underlying pathophysiology and pathogenesis. Recognition of the role of infection and inflammation in these disorders is gaining momentum; bacterial gastroenteritis, for example, constitutes the highest risk factor identified to date for the development of irritable bowel syndrome.[2] Biopsy specimens from patients with postinfective irritable bowel syndrome provide evidence of ongoing immune activation[3] and indicate that the immune response to the initiating infection is a critical determinant in the development of persistent gut dysfunction.[4]

Like irritable bowel syndrome, the pathophysiology of functional dyspepsia is poorly understood; abnormal motility, visceral hypersensitivity, and psychological factors have been implicated.[5] However, the role of infection and inflammation in functional dyspepsia remains controversial. The controversy focuses on the role of *Helicobacter pylori* infection. Demonstrations of altered gastroduodenal motility and sensory function have been documented in some[6–11] but not all[12–14] dyspeptic patients with chronic *H. pylori* infection. Furthermore, eradication of the infection is not always accompanied by symptomatic improvement, particularly in the short-term.[15–19] Recent meta-analyses of the benefit of *H. pylori* eradication on symptom improvement in functional dyspepsia have yielded conflicting results.[20,21] Thus, the role of *H. pylori* infection in functional dyspepsia remains an active and controversial field.

H. pylori colonizes the surface of the gastric epithelium and induces mucosal changes, chronic gastritis, and both a systemic and local immune response.[22] The latter may be quite variable in intensity and persistence following successful eradication.[23] A recent study has suggested that patients with functional dyspepsia and a more chronic lymphocytic infiltrate on gastric biopsies respond poorly to eradication compared with those with a more prominent acute inflammatory response.[24] Thus, the chronicity of the infection and its accompanying immune

Abbreviations used in this paper: ACh, acetylcholine; EFS, electric field stimulation; IR, immunoreactivity; MN, mononuclear; PMN, polymorphonuclear.

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response may be a determinant of altered gastric physiology, symptom generation, and responsiveness to eradication.

The purpose of this study was to use a murine model of chronic *H. pylori* infection to determine whether infection generates changes in gastric neuromuscular physiology and to evaluate the dependence of these changes on the degree of bacterial colonization, the severity of the chronic inflammatory infiltrate, and the immune response to infection. Our results indicate that chronic *H. pylori* infection is accompanied by changes in neural circuitry, including sensory nerves. We show that these changes are related to the inflammatory cell infiltrate rather than degree of bacterial colonization. Of potential importance to current clinical research, we show that these changes are critically dependent on the immune response and may not reverse rapidly following *H. pylori* eradication. These results provide insights into the basis of the controversy that currently exists regarding the role of *H. pylori* in functional dyspepsia and the role of eradication therapy.

Materials and Methods

Bacteria

H. pylori (Sydney strain) was cultured for 24 hours in liquid media derived from brain-heart infusion (BBL, Sparks, MD) containing 0.3% yeast (Difco Laboratories, Detroit, MI) and 10% bovine serum (Gibco BRL, Grand Island, NY) and supplemented with 0.4% *Campylobacter* selective complement (Skirrow supplement SR69; Oxoid, Basingstoke, England) under a microaerophilic atmosphere at 37°C.

Animals

The experimental procedure was approved by the Animal Welfare Committee of McMaster University Medical Center. Specific pathogen–free (*Helicobacter* species) female Balb/c mice (Harlan, Indianapolis, IN) and C.B-17 (lcrHsd-SCID) mice (Harlan) that were 6–8 weeks old were infected by intragastric gavage with *H. pylori* at a dose of 10^9 bacteria per mouse on 3 separate occasions, each 48 hours apart.

After the mice were killed, the abdomen was opened by midline incision and the stomach was isolated and cut along the greater curvature. The gastric contents were gently washed with saline, and 2 antral strips (3 × 10 mm) were taken for acetylcholine (ACh) superfusion or muscle contractility experiments. A longitudinal section of the stomach along the lesser curvature was placed in 10% formalin fixative for histologic examination. The remaining gastric tissue and the thoracolumbar spinal cord were placed in 4% paraformaldehyde fixative for immunohistochemistry.

Experimental Design

Experiments were performed in uninfected mice as well as in mice at 2 weeks after infection (acute infection) and at 3–16 months after infection (chronic infection). An additional group of mice infected for 10 months received eradication therapy consisting of pantoprazole (0.62 mg/kg body wt), amoxicillin (15.4 mg/kg body wt), and clarithromycin (7.7 mg/kg body wt) twice daily for 10 days. Experiments were then performed 2 months after eradication treatment. All experiments were performed in Balb/c mice. Additional experiments on ACh release, severity of gastritis, *H. pylori* colonization, and macrophage density were performed in uninfected SCID mice and in SCID mice 7 months after infection.

Histology

Paraffin sections of gastric tissue were stained with H&E to grade for gastritis and with Warthin–Starry stain for bacterial colonization. Polymorphonuclear (PMN) and mononuclear (MN) cell infiltration were graded separately in the corpus and antrum on a scale of 0–3 as described by Shirai et al.[25] Bacterial colonization was graded on a scale of 0–4 according to Lee et al.[26]

Immunohistochemistry for Macrophages

Immunohistochemistry was performed on paraffin sections using a monoclonal antibody recognizing F4/80 antigen, which is expressed by murine macrophages and monocytes. We used a modified version of a previously described method for analysis of frozen sections.[27] Briefly, after blocking endogenous peroxidase by 1.5% methylated peroxide for 25 minutes, the slides were immersed in trypsin solution for 10 minutes. After washing with Tris-buffered saline, the endogenous biotin was blocked by adding avidin and then biotin solutions (Vector, Burlington, Canada), both for 15 minutes. After washing in Tris-buffered saline, the slides were incubated overnight with rat anti-mouse antibody F4/80 (1:800 dilution; Serotec, Oxford, England). The slides were then washed in Tris-buffered saline, and biotinylated polyclonal goat anti-rat antibody (1:200 dilution; Cederlane Laboratories, Hornby, Canada) that was previously incubated with 10% mouse serum was added for 1 hour. After washing with Tris-buffered saline, streptavidin/horseradish peroxidase (Gibco BRL, Life Technologies, Burlington, Canada) was added at 1:300 dilution for 1 hour. The slides were washed with Tris-buffered saline, and the antibodies were visualized by 3-amino-9-ethylcarbazole. The tissues were counterstained with Mayer's hematoxylin and examined by light microscopy. Macrophages were counted in the submucosa and muscularis externa using modified score of Shirai et al.[25] for MN cells.

Immunohistochemistry for Substance P–, Calcitonin Gene-Related Peptide–, and Vasoactive Intestinal Polypeptide–Containing Nerves

Stomach and spinal cord (lower thoracolumbar region) tissues fixed in 4% paraformaldehyde for 12 hours were subsequently placed in 25% sucrose until sectioning. Cryosections were processed with the avidin-biotin-peroxidase complex method as described previously.[28,29] We used rabbit polyclonal anti–calcitonin gene-related peptide $(CGRP)_{1-37}$ (1:2000 dilution), anti–substance P (SP; 1:5000 dilution), and anti–

vasoactive intestinal polypeptide (VIP; 1:5000 dilution) as primary antibodies and purified goat anti-rabbit biotinylated immunoglobulin G (1:100 dilution; Vector Laboratories, Burlingame, CA) as secondary antibodies. In control experiments, the primary antibodies were either substituted with normal rabbit serum (dilution 1:50) or incubated for 12–16 hours with synthetic homologous or heterologous peptides (Tyr-rat $CGRP_{23–37}$, rat $CGRP_{1–37}$, SP, and VIP at concentrations of 10 μmol/L; Bachem [Torrance, CA] or Peninsula Laboratories [Belmont, CA]). Because the immunoreactive materials could be structurally related but not identical to the peptides under investigation, the terms CGRP-, SP-, and VIP-immunoreactivity (IR) are used to describe the immunostaining. Quantification of IR was performed using a Leitz (Weitzlar, Germany) Orthoplan microscope (25× objective) by 2 operators in a blind fashion using a computer-assisted analysis system (Cytometrica software; C&V, Bologna, Italy) by a modification of previously published methods.[30–32] Briefly, whole gut or spinal cord cross sections were randomly sampled with the aid of a grid (0.5 mm squares) located below the slide. Once the immunoreactive threshold was set, the computer program binarized the image and selected nerve fibers with staining intensity sufficiently distinct from the background. The immunoreactive nerve fiber density was expressed in pixels per optical field.

Longitudinal Muscle Contractility

Measurement of muscle contraction was performed as described previously.[33] Briefly, muscle strips suspended in muscle bath containing oxygenated Krebs solution at 37°C were stretched by tension equivalent to 0.5 mg, and contractility of the longitudinal muscle was recorded after a 40-minute equilibration period by force transducers (FT03C; Grass, Quincy, MA) connected to a polygraph (model 7D; Grass). Contractility was assessed by electric field stimulation (EFS) at muscle (50 V, 10 Hz, 5 milliseconds) or nerve (50 V, 10 Hz, 0.5 millisecond) parameters or pharmacologically by adding 50 mmol/L KCl or 10^{-5} mol/L carbachol. The force generated by the muscle strips was expressed in milligrams and normalized for cross-sectional area.

ACh Release

A technique to measure [^{3}H]ACh release in rat intestine[34] was adapted to the mouse stomach. Briefly, antral strips equilibrated previously for 40 minutes in oxygenated Krebs buffer containing 0.2 μmol/L [^{3}H]choline (New England Nuclear, Boston, MA) were suspended in a superfusion chamber at 37°C and superfused with Krebs buffer containing 10 μmol/L hemicholinium. The release of ACh was stimulated by EFS (30 V, 10 Hz, 0.5 millisecond) or 50 mmol/L KCl, and the superfusate was collected every 2 minutes. After adding counting fluid, ^{3}H content was measured by a Beckman (Irvine, CA) scintillation counter (LS 5801). At the end of the experiment, the tissues were blotted dry and solubilized by NCS solution (Amersham, Oakville, Canada). After neutralization by acetic acid, counting fluid was added and ^{3}H content was measured. Stimulated [^{3}H]ACh release was calculated as the difference between the total [^{3}H]ACh released during the stimulation and the extrapolated spontaneous outflow and was expressed as a percentage of the total [^{3}H]ACh present in the tissue.

Statistics

Because the data did not follow a normal (parametric) distribution in most cases, medians and interquartile ranges are presented. The figures are presented as box-whiskers plots in which the box shows the interquartile range, the horizontal line shows the median (50th percentile), and the whiskers show the 5th and 95th percentiles. Statistical testing was performed using the Mann–Whitney test for nonparametric unpaired data. The strength of association between parameters was evaluated using linear correlation and the Spearman rank correlation tests. A P value <0.05 was considered statistically significant.

Results

Histology

Animals infected with *H. pylori* for 2 weeks showed moderate to high bacterial colonization, and a higher grade was seen in the corpus compared with the antrum. This was accompanied by a mild active gastritis

Table 1. Histology Results

	Controls	2 wk	3–8 mo	9–16 mo	Posteradication
Colonization	0.0 (0.0)	2.2 (1.0)[a]	2.0 (1.0)[a]	2.0 (1.5)[a]	0.0 (0.0)[b]
PMN/corpus	0.0 (1.0)	1.0 (0.5)[a]	1.0 (1.7)[a]	1.5 (1.0)[a]	0.5 (1.2)[b,c]
PMN/antrum	0.0 (0.0)	2.0 (0.5)[a]	1.0 (1.0)[a]	1.0 (1.0)[a]	0.7 (1.0)[c]
MN/corpus	0.0 (1.0)	1.0 (0.5)[a]	2.0 (1.0)[a]	2.5 (1.2)[a]	1.5 (0.7)[a,b]
MN/antrum	0.0 (1.0)	1.0 (0.5)[a]	1.0 (1.0)[a]	1.2 (1.0)[a]	1.0 (0.5)[a]
Macrophages/corpus	0.0 (0.0)	0.7 (0.7)[a]	1.0 (1.0)[a]	2.0 (1.8)[a]	NA
Macrophages/antrum	0.0 (0.0)	0.0 (0.2)	0.0 (0.2)	1.0 (1.1)[a]	NA

NOTE. Results are expressed as median (interquartile range) in units on a scale of 0–4 for *H. pylori* colonization and 0–3 for the remaining parameters.
[a]$P < 0.01$ vs. controls.
[b]$P < 0.05$ vs. 12–16 months.
[c]$P < 0.05$ vs. controls.

consisting of a predominantly acute inflammatory infiltrate (PMN) that was greater in the antrum (Table 1). At 3–8 months after infection, bacterial colonization was moderate and limited to the gastric corpus and to the transitory zones between the corpus and cardia and between the corpus and antrum. A predominantly MN infiltrate was present in the corpus, whereas the PMN infiltrate was less in the antrum. Immune cells were mainly localized in the submucosal layer, forming occasional lymphocytic aggregates in the corpus (Figure 1*B*). At 9–16 months after infection, the MN infiltrate was dense and more pronounced in the corpus, creating large lymphocytic aggregates (Figure 1*C*). The pattern of the inflammatory infiltrate in the antrum and bacterial colonization remained unchanged. The MN infiltrate in the muscularis externa and myenteric plexus progressively increased during the course of the infection from an occasional MN cell in controls to 20–30 cells per visual field in mice infected for 9–16 months. After successful eradication of *H. pylori* infection, the gastritis score was significantly reduced in the corpus (Table 1).

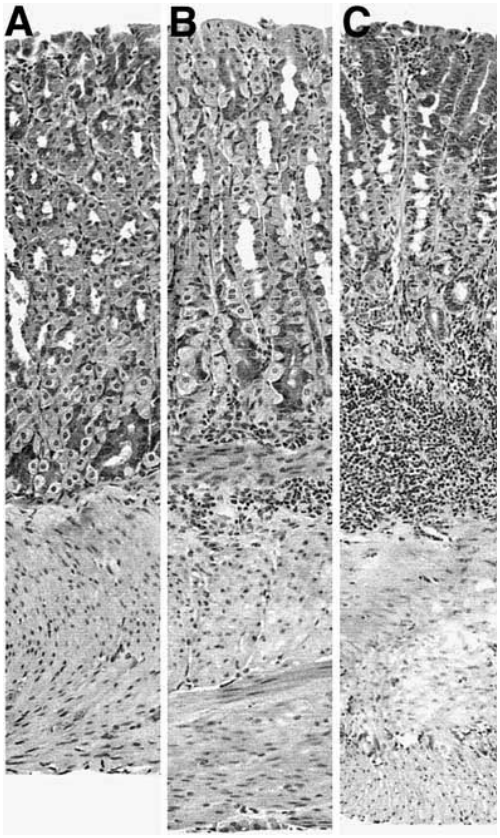

Figure 1. Representative H&E staining of gastric corpus (*A*) from an uninfected Balb/c mouse, (*B*) 4 months after infection, and (*C*) 12 months after infection. A predominantly MN infiltrate is present in the mucosa and submucosa (*B*) with lymphoid aggregates in later stages of the infection (*C*).

Table 2. Muscle Contractility

Stimulation	Controls (n = 16)	2 wk (n = 6)	3–8 mo (n = 17)
Carbachol	338.6 (344.0)	474.8 (365.5)	250.8 (173.9)
KCl	144.7 (260.5)	311.8 (472.1)[a]	149.1 (132.1)
EFS (muscle parameters)	68.8 (104.9)	164.2 (402.1)[b]	70.4 (173.9)

NOTE. All values are expressed in mg/mm^2. All comparisons are nonsignificant unless otherwise noted.
[a]$P = 0.06$ vs. controls.
[b]$P < 0.05$ vs. controls.

Muscle Contractility

Carbachol- and KCl-stimulated contractility tended to be higher 2 weeks after infection than in controls but not 3–8 months after infection (Table 2). Contractility induced by EFS at muscle parameters was higher in infected than in control mice at 2 weeks but was similar 3–8 months after infection (Table 2). Eradication treatment did not affect EFS or pharmacologically stimulated muscle contractility (data not shown).

Stimulation of antral strips by EFS at neural parameters caused muscle relaxation. The relaxation was greater 2 weeks and 3–8 months after infection than in controls (−65.2 [42.6], −86.7 [116.9], and −30.9 [47.8] mg/mm^2, respectively; $P < 0.05$ vs. controls). Two months after eradication of the infection, the EFS-induced relaxation normalized, reaching a median value of −22.8 (30.6) mg/mm^2 (Figure 2).

ACh Release in Balb/c Mice

EFS-induced ACh release was 0.54 (0.58)% in controls and 0.39 (0.79)% 2 weeks after infection and

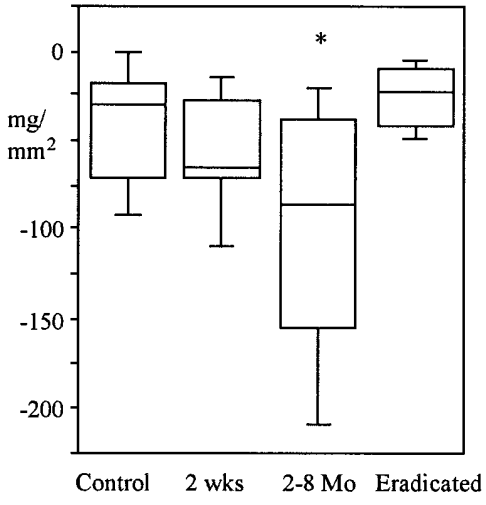

Figure 2. EFS of enteric nerves induced antral muscle relaxation in control mice. Chronic *H. pylori* infection augmented this relaxation. Two months after eradication therapy, EFS-induced relaxation normalized. Data are presented as medians and interquartile ranges. *$P <$ 0.05 vs. control.

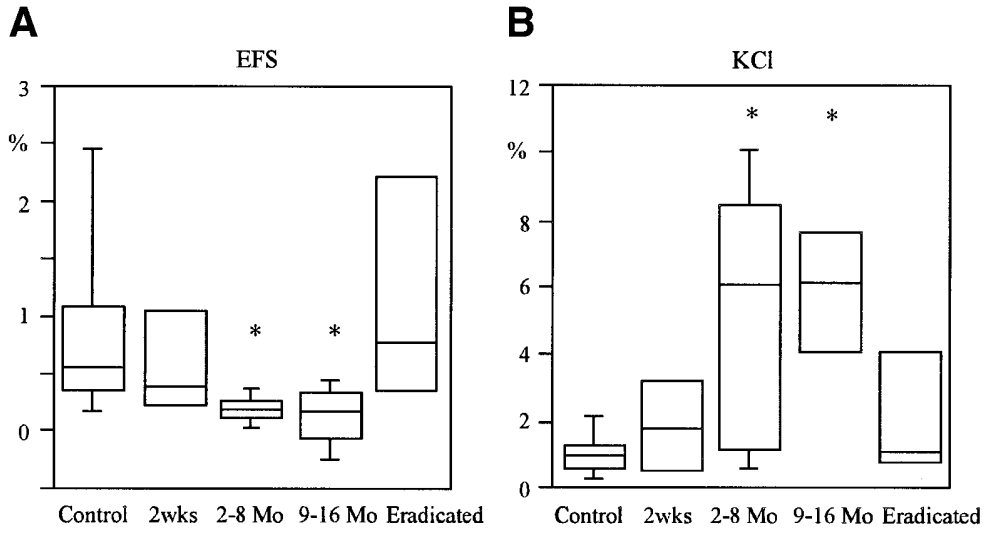

Figure 3. ACh release after EFS and KCl stimulation. Chronic *H. pylori* infection progressively decreased (*A*) EFS-induced ACh release but (*B*) increased KCl-induced ACh release. Eradication therapy normalized the function of cholinergic nerves. $*P < 0.05$ vs. control.

decreased to 0.18 (0.24)% 3–8 months after infection ($P = 0.002$ vs. control) and to 0.13 (0.24)% 9–16 months after infection ($P = 0.002$ vs. control) (Figure 3). Eradication of the infection normalized EFS-induced ACh release to a median of 0.79 (1.50)%.

KCl-stimulated release was 0.96 (0.77)% in controls and increased to 1.82 (2.66)% 2 weeks after infection, 6.14 (7.39)% 3–8 months after infection ($P = 0.02$ vs. control), and 6.18 (3.59)% 9–16 months after infection ($P = 0.003$ vs. controls) (Figure 3). Eradication also normalized KCl-induced ACh release to 1.05 (3.27)%.

SCID Mice

To determine whether these changes in gastric neural function are dependent on the immune response to infection, we performed studies in SCID mice, which lack T and B lymphocytes. SCID mice infected for 7 months showed a moderate to high degree of bacterial colonization and a mild gastritis consisting of occasional PMN as well as moderate macrophage infiltrate that was confirmed by immunohistochemistry. However, EFS-induced ACh release was 0.26 (0.32)% in controls and 0.30 (0.45)% in *H. pylori*–infected mice ($P = 0.62$). Similarly, KCl-stimulated ACh release was 0.23 (0.41)% in controls and 0.58 (0.39)% in *H. pylori*–infected mice ($P = 0.15$).

Density of SP-, VIP-, and CGRP-IR Nerves in the Stomach

In control mice, SP-IR nerves were densely distributed in the muscle layers around unstained or, more frequently, stained ganglion cell bodies of the myenteric plexus and around the vasculature of the submucosa. SP-IR nerves were also found in the mucosa in close association with the gastric glands and with the muscularis mucosae. CGRP-IR nerves were observed throughout the muscle layers, some of which were in close contact with unstained myenteric ganglion cell bodies. A particularly dense CGRP nerve supply was seen in association with blood vessels in the submucosa. CGRP-IR nerves were identified throughout the gastric glands. VIP-IR nerves were abundantly present throughout the muscle layers and supplied stained and unstained perikarya of myenteric neurons. VIP-IR nerves were visualized in the submucosa, muscularis mucosae, and mucosa, where labeled fibers closely surrounded gastric glands.

The overall distribution of peptide-labeled nerves was unchanged by *H. pylori* infection. However, the density of SP-IR nerves increased from 1056 (974) pixels per field in controls to 2559 (1451) in animals infected for 3–8 months ($P = 0.04$). After eradication of the infection, the values decreased to 1830 (1019) but remained higher ($P = 0.06$) than in controls (Figure 4). The density of CGRP-IR nerves increased from 1165 (619) pixels per field in controls to 2237 (1317) in animals infected for 3–8 months ($P = 0.03$). After eradication, the values decreased to 1765 (989) but remained higher ($P = 0.06$) than in controls (Figure 4). The density of VIP-IR nerves increased from 1496 (281) pixels per field in controls to 2789 (638) in animals infected for 3–8 months ($P = 0.02$). In contrast to SP- and CGRP-, VIP-IR nerves returned to normal with values of 1523 (585) (Figure 4) after eradication of the infection.

Density of SP- and CGRP-IR Nerves in the Spinal Cord

A dense network of immunoreactive SP and CGRP varicose nerve fibers was identified in the dorsal

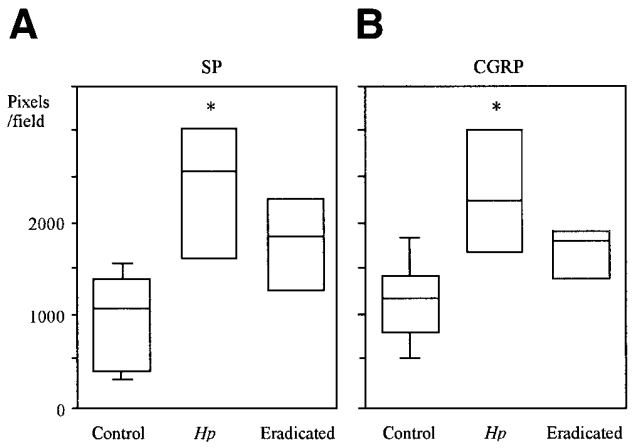

Figure 4. Chronic *H. pylori* infection increased the density of (*A*) SP-IR nerves and (*B*) CGRP-IR nerves in the stomach. Two months after eradication treatment, the density of SP- and CGRP-IR nerves remained elevated. $*P < 0.05$ vs. control.

horn of the spinal cord, whereas VIP-IR was absent. SP- and CGRP-IR varicosities were largely restricted to laminae II and I of the dorsal horn. Occasionally, CGRP varicosities were also found in the innermost portions of the spinal cord. No peptide-containing neuronal cell bodies were identified in these preparations.

The density of SP-IR nerves increased from 1056 (974) in controls to 2559 (1451) pixels per field in animals infected for 3–8 months ($P = 0.04$). Eradication of the infection normalized the values to 1830 (1019) pixels per field (Figure 5). Similarly, the density of CGRP-IR nerves increased from 1165 (619) pixels per field in controls to 2237 (1317) in animals infected for 3–8 months ($P = 0.03$) and returned to normal with values of 1765 (989) 2 months after eradication (Figure 5).

Immunohistochemistry of Macrophages

Occasional macrophages were present in the mucosa and submucosa of uninfected Balb/c mice (Figure 6*A*). Macrophage infiltration was high 2 weeks after infection, further increased at 3–8 months in the corpus (Figure 6*B*), and peaked at 9–16 months (Figure 6*C* and Table 1). Macrophages were mainly present in the submucosa as part of an extensive chronic infiltrate, but some were present in the muscularis externa (between 10 and 20 positive cells per visual field) 9–16 months after infection.

In uninfected SCID mice, occasional macrophages were also present in the mucosa and submucosa. The macrophage infiltration scores in SCID mice significantly increased 7 months after infection, reaching values similar to those observed in Balb/c mice at comparable time points (1.2 [1.0] and 1.0 [0.7] in the corpus and antrum, respectively). In contrast to Balb/c mice, macrophages in SCID mice were almost restricted to the mucosa and submucosa, with only 2–3 cells per visual field in the muscularis externa.

Correlation Between ACh Release and Inflammatory Response to *H. pylori*

To investigate the relationship between impaired ACh release and immune response to *H. pylori*, we plotted ACh release values from control and infected mice against the level of *H. pylori* colonization as well as PMN and MN infiltrate. EFS-induced ACh release correlated with the degree of MN infiltrate both in the antrum ($r = 0.47$, $P = 0.009$) and corpus ($r = 0.50$, $P = 0.004$) (Figure 7) but not with the degree of PMN infiltrate or with bacterial colonization. Similarly, KCl-stimulated ACh release correlated with the degree of MN infiltrate both in the antrum ($r = 0.45$, $P = 0.01$) and corpus ($r = 0.49$, $P = 0.009$) but not with the degree of PMN infiltrate or with bacterial colonization.

Discussion

We have shown for the first time that chronic *H. pylori* infection induces functional and morphologic changes in gastric neural circuitry in mice. These changes involve impaired antral relaxation and ACh release as well as altered content of sensory neurotransmitters in the stomach and spinal cord. Moreover, we show a role for the immune response to *H. pylori*, because the observed neural abnormalities were absent in infected SCID mice. The functional changes worsen during the course of the infection, and some persist after the bacterial eradication.

There is growing evidence that infectious agents play a role in the pathogenesis of many human chronic inflammatory disorders. An abnormal immune activation to intestinal bacteria that may be genetically determined is supported by clinical and experimental data in inflammatory bowel disease and more recently in extraintestinal systems such as rheumatoid arthritis.[35–38] Respiratory tract infections have also been implicated in immune activation and development of airway hyperreactivity and asthma.[39]

Previous experiments in animal models of inflammation have clearly shown a relationship between the immune and neuromuscular systems in the gastrointestinal tract. Muscle cells have been shown both as targets and active participants of the inflammatory response in *Trichinella spiralis* infection.[40] We have observed increased stimulated muscle contractility during acute *H. pylori* infection, but chronic infection did not affect muscle function per se. The observed transient muscle hy-

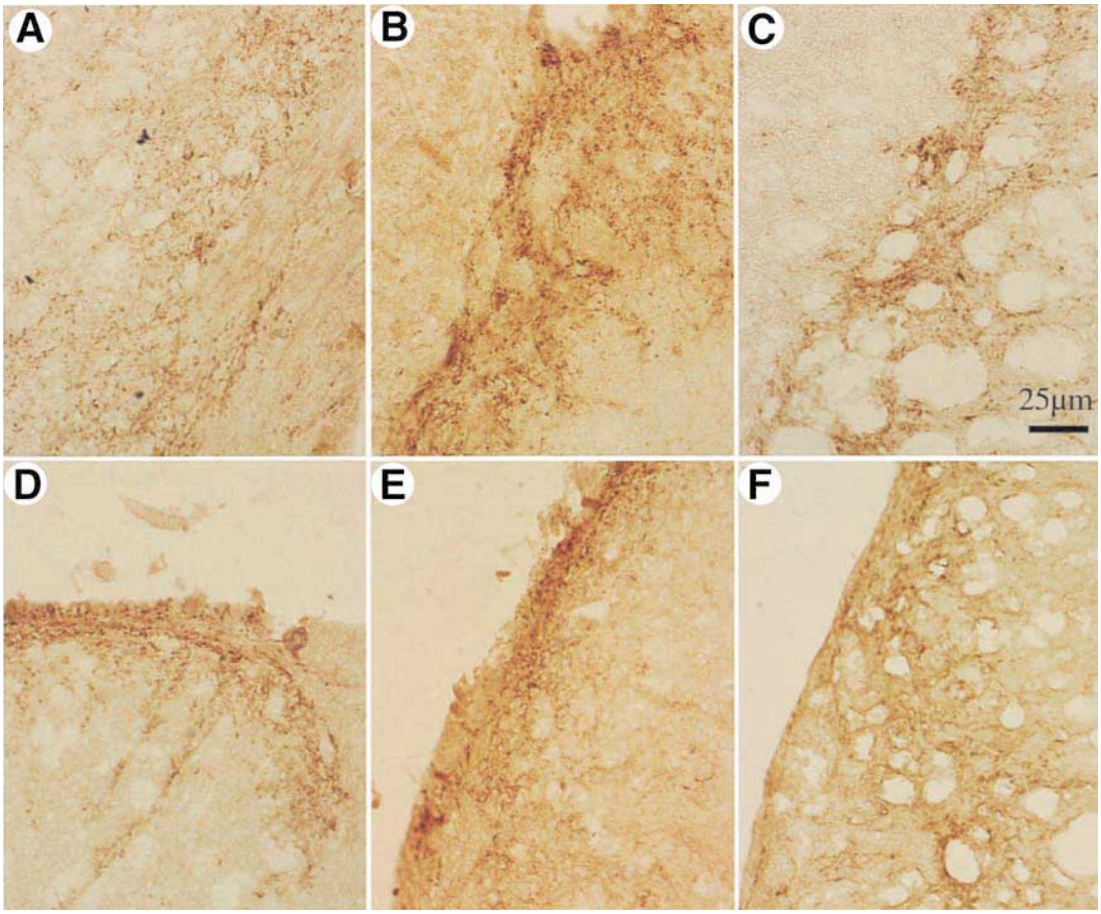

Figure 5. (*A–C*) Representative photomicrographs showing SP-IR nerves in the spinal cord from (*A*) an uninfected mouse, (*B*) a mouse 10 months after infection, and (*C*) a mouse 2 months after eradication of *H. pylori*. (*D–F*) Representative images of CGRP-IR nerves in the spinal cord from (*D*) an uninfected mouse, (*E*) a mouse 10 months after infection, and (*F*) a mouse 2 months after eradication of *H. pylori*.

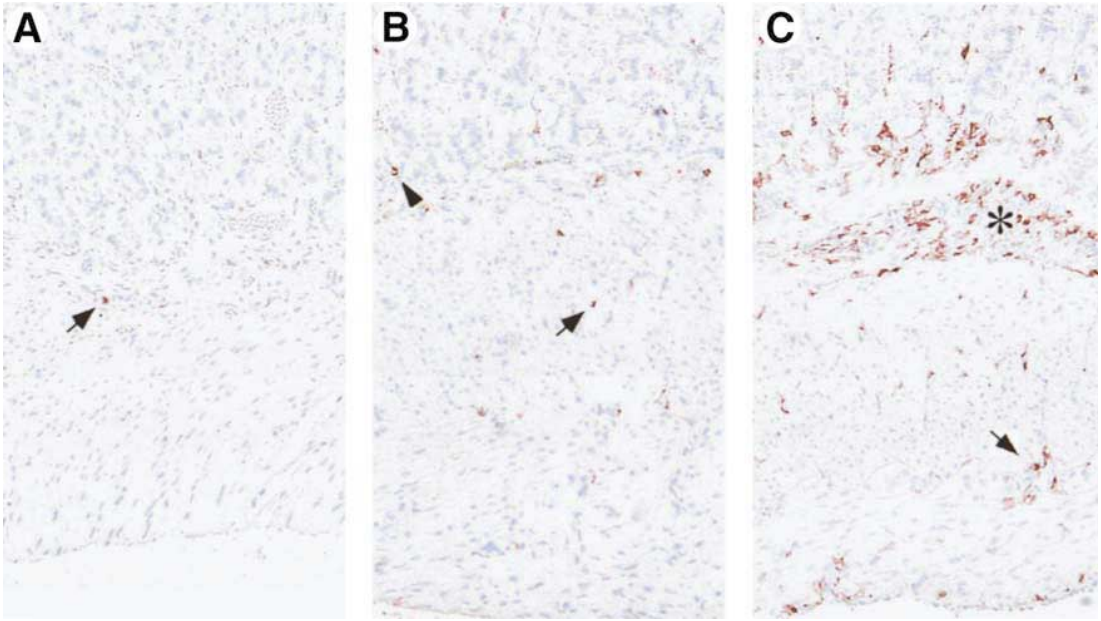

Figure 6. (*A*) Occasional F4/80-positive cells (*arrow*) in the submucosa of an uninfected Balb/c mouse. (*B*) F4/80-positive cells appear both in the mucosa (*arrowhead*) and the muscle layer (*arrow*) in a mouse 4 months after infection. (*C*) At 12 months after infection, the number of F4/80-positive cells further increases, many of them being part of the chronic inflammatory infiltrate in the submucosa (*). Macrophages are also present at the region of the myenteric plexus (*arrow*).

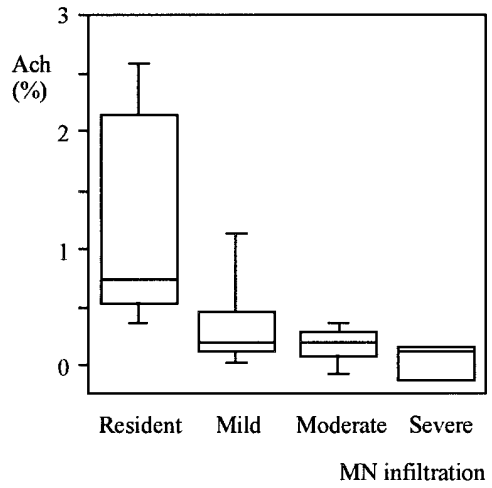

Figure 7. Impairment of ACh release after EFS correlates with the degree of MN infiltration of the stomach.

percontractility may be thus related to increased PMN infiltration during the acute phase of the infection.

We induced muscle relaxation when stimulating nerves in antral strips with EFS, indicating that antral muscle is under tonic neural inhibitory control. Antral relaxation was greater during chronic *H. pylori* infection, suggesting an increase in neural inhibition, a decrease in neural excitation, or both.

Levels of ACh, a major excitatory neurotransmitter in the gut, have been shown to be altered in different models of inflammation.[34,41] In our study, EFS-induced ACh release was lower during chronic *H. pylori* infection, which suggests decreased synthesis of the neurotransmitter, impaired storage, or altered release. In contrast to EFS, which reflects the functional status of cholinergic nerves, KCl administration via neuronal depolarization releases all available ACh present in the tissue. KCl-stimulated ACh release was higher in infected animals than in controls. It is therefore unlikely that the lower EFS-induced ACh levels were due to impaired synthesis or storage. An altered pattern of release may result from the functional reorganization of the enteric nervous system, similarly to what has been recently reported in nematode infection,[42] with a resulting higher inhibitory drive. The impaired cholinergic nerve function might then explain the greater degree of antral muscle relaxation observed after neural EFS stimulation in infected animals by reducing the excitatory input.

VIP is one of the main inhibitory neurotransmitters acting on intestinal muscle, and its level is altered during experimental gut inflammation.[43,44] We found that chronic *H. pylori* infection caused a 2-fold increase in the density of VIP-IR nerves in the stomach without affecting the distribution pattern of this peptide. The greater antral relaxation observed in infected animals may therefore be attributed in part to an increased level of this neurotransmitter. In addition to VIP, nitric oxide is a major inhibitory neurotransmitter in the gastrointestinal tract. It has been shown that neuronal NO synthase–deficient mice develop dilation of the stomach and delayed gastric emptying.[45] It is possible that alteration of NO-containing neurons contributes to the increased antral relaxation observed in our experiments.

Altered sensory perception is considered a major pathophysiologic mechanism in functional gastrointestinal disorders.[46] Inflammation may affect the sensory system by different mechanisms, including peripheral and central sensitization processes, as well as via changes of gene expression in the dorsal root ganglia and dorsal horn neurons.[47] SP and CGRP are the main sensory and proinflammatory neurotransmitters in the gut. Increase in SP content in gut has been reported during *T. spiralis* infection[48] as well as after incubating the intestinal tissue with interleukin 1β,[49] a cytokine produced by several MN cell types. We have observed a 2.5- and 2-fold increase in the density of SP- and CGRP-IR nerves, respectively, both in the gastric wall and dorsal horn of the spinal cord of infected animals. It is plausible that these morphologic alterations result in functional disturbances of the sensory system. In support of this notion, a recent study has shown that gastric pain thresholds correlate with mucosal concentrations of SP and CGRP in patients with functional dyspepsia.[50]

To determine whether neural abnormalities are related to the bacterial colonization or the associated inflammation, we correlated cholinergic nerve dysfunction with different aspects of the infection. Bacterial colonization was highest during the acute infection and remained stable thereafter. PMN infiltrate in the antrum also peaked during acute infection and remained elevated both in the antrum and corpus throughout the course of the infection. In contrast, MN infiltrate, consisting mainly of lymphocytes, gradually increased and peaked 9–16 months after infection, following the same trend as the impaired ACh release. Also, both EFS and KCl stimulated ACh release, correlated with the degree of MN infiltrate. Furthermore, no cholinergic nerve impairment was observed in infected SCID mice, which lack both T and B lymphocytes but retain moderate to high *H. pylori* colonization and macrophage infiltration. Thus, our results indicate that the neural dysfunction is related to the inflammatory response rather than the degree of colonization. In this regard, it is of interest to note that functional dyspepsia is associated with infection by *H. pylori* strains[51–53] that induce a more severe gastritis[54,55] and that the resolution of gastritis after *H. pylori* eradication was a predictor of symptomatic improvement in

patients with functional dyspepsia.[18] In addition, a lower threshold for duodenal distention was found in dyspeptic patients with higher antibody titers against *H. pylori* than in those with low levels of antibodies,[10] which further supports the role of the immune system in functional abnormalities during *H. pylori* infection.

Neuronal changes in the model of *T. spiralis* infection are T cell independent[34] and involve macrophages.[27] In the *H. pylori*–infected mouse, our results indicate that the changes in neural function are dependent on the inflammatory response and on some component of the immune response absent in SCID mice. The lack of T-cell dependence of neural changes in the nematode model contrasts with an apparent T-cell dependence in *H. pylori*–infected mice. This discrepancy may reflect differences in the nature of the underlying immune response (Th2 in nematode infection compared with Th1 in *H. pylori* infection). In addition, the current study is of a chronic infection, whereas the nematode study was an acute model. Distribution of macrophages may be another important factor; *H. pylori*–infected SCID mice, despite a similar overall level of macrophage infiltration in the mucosa and submucosa, had very few macrophages in the muscularis externa. Thus, in contrast to the *T. spiralis* model, if macrophages are involved in the neuronal damage during chronic *H. pylori* infection, they require T lymphocytes for their activation.

H. pylori eradication normalized cholinergic nerve function, antral relaxation, the increased density of SP- and CGRP-IR nerve fibers in the spinal cord, and the density of VIP-IR fibers in the stomach. However, the density of SP- and CGRP-IR nerves remained elevated in the stomach 2 months after the eradication treatment. These results show the plasticity of the enteric nervous system in this model and that some of the changes are slowly reversible. This may have bearing on clinical studies showing delayed symptomatic benefit following *H. pylori* eradication in patients with functional dyspepsia.

Our results are relevant to the clinical controversy surrounding the role of *H. pylori* infection in functional dyspepsia. First, our demonstration of alterations in efferent and sensory nerves provides proof of the concept that chronic *H. pylori* infection alters gastric physiology and could be a basis for symptom generation in infected patients with functional dyspepsia. Second, we show that altered neural function is immune dependent and related to the degree of gastritis rather than the degree of bacterial colonization. This concurs with a recent clinical observation linking symptoms to the severity of gastritis.[24] Finally, we show that not all neural changes revert immediately following eradication of *H. pylori.* This may account for the failure to show symptomatic improvement in patients in the short-term after eradication and the trend toward an improvement in those patients reviewed at late time points after *H. pylori* eradication. Based on these findings, reconsideration of the role of *H. pylori* in functional dyspepsia is recommended.

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