**Helicobacter Pylori-associated Gastric Cancer in INS-GAS Mice Is Gender Specific**

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**ABSTRACT**

Previous studies from our group have shown that hypergastrinemia in mice can synergize with *Helicobacter felis* infection to induce gastric carcinoma. In addition, epidemiological evidence and a recent study with C57BL/6 mice have strongly suggested a link between a high-salt diet during *Helicobacter pylori* infection and the development of hypergastrinemia and preneoplastic gastric lesions. To address the possible relationship between the two cofactors (gastrin and salt) and whether *H. pylori* can also lead to gastric cancer in this model, we undertook a longitudinal study involving 86 INS-GAS mice. The mice were fed either a high-salt (7.5%) or basal (0.25%) diet, and half were infected with *H. pylori*. Necropsies at 5 and 7 months postinfection included histopathological examination, quantitative culturing for bacterial colonization levels, and serology to estimate the magnitude of the Th1 and Th2 systemic inflammatory responses. Lesions consistent with *in situ* and intramucosal carcinoma were seen in *H. pylori*-infected male mice only. There was a highly significant main effect for *Helicobacter* infection status for all fundic and antral lesion parameters (*P* < 0.0001), as well as significant interactions of infection status with diet for all of the fundic parameters (all *P* < 0.03), except intestinal metaplasia. In subsequent ANOVAs in which the data were limited to that from infected animals, there was a highly significant main effect for time, diet, and gender (all *P* < 0.02) on all of the corpus lesion parameters scored (inflammation, atrophy, hyperplasia, metaplasia, and dysplasia/neoplasia). In addition, gender interacted significantly with time (all *P* < 0.03), and *H. pylori* colonization increased quantitatively over the course of the experiment but were independent of either diet or gender. The Th1-associated serum IgG2a responses to *H. pylori* increased from the time of experimental infection to necropsy at 5 or 7 months and were similar among all experimentally infected mice with no influence of gender (P > 0.10) or dietary salt (*P* > 0.27). In contrast, the Th2-associated serum IgG1 response to *H. pylori* was significantly increased in infected male INS-GAS mice on the high-salt diet at 7 months postinfection (*P* < 0.012). These results show that *H. pylori* can also accelerate the development of gastric cancer in the INS-GAS mouse model, and the results suggest that salt has less of a procarcinogenic effect in the setting of endogenous hypergastrinemia. The increased Th2-associated humoral response of the infected male mice on the high-salt diet correlated with less severe gastric lesions. In the INS-GAS mouse model, male gastric tissue responded more rapidly and aggressively to *H. pylori* infection, high-salt diet, and the combination when compared with females; a finding that appears consistent with the greater incidence of gastric carcinoma in men. This study highlights the importance of using both genders to investigate the pathogenesis of *H. pylori*.

**INTRODUCTION**

Both tumor initiators as well as tumor promoters are thought to play an important role in gastric carcinogenesis. The deleterious effects of salt, including its role in gastric cancer, is based on epidemiological studies, biochemical analyses, and *in vivo* experimentation (1, 2). An increased risk of gastric cancer has been associated with the ingestion of salty preserved food (3), as well as preference for salty foods (4, 5), which incorporate the measurement of salt consumption (6–9). Salt administered p.o. on a weekly basis or fed in the diet during 12–20 weeks of exposure to MNNG3 in drinking water promoted the induction of gastric adenocarcinoma in rats (10–12). Ingestion of salt by rats leads to chronic injury of the surface gastric epithelium followed by gastric epithelium proliferation (13–16). Rats given salt in the diet and MNNG in drinking water developed an 80% incidence of adenocarcinomas in the antrum but not in the corpus (11), whereas rats given a single dose of MNNG [MNNG in water (5 g/liter), 0.25 ml/10 g body weight] by intubation and fed a 10% NaCl diet for 1 year had significantly increased tumor burden in both the forestomach and glandular stomach (17). Mice fed a rice diet containing highly salted food developed acute gastric mucosal damage (18). Swiss/ICR mice fed salted (10% w/w NaCl) rice diets for 3–12 months developed hypertrophy of the forestomach and atrophy (noted by a loss of parietal cells) of the glandular stomach (19).

We recently fed a high-salt diet (7.5% versus 0.25%) for 16 weeks to female C57BL/6 mice infected with *H. pylori* (20). Half of the infected and control mice were fed the high-salt diet for 2 weeks before dosing and throughout the 16-week experiment. At 16 WPI, mice in both the normal and the high salt diet groups developed moderate to marked atrophic gastritis of the corpus in response to *H. pylori* infection. Also at 8 and 16 WPI, cfu/g tissue of *H. pylori* were significantly higher (*P* < 0.05) in the corpus and antrum of animals in the high-salt diet group compared with those on the normal diet. The gastric pits of the corpus mucosa in mice on the high-salt diet were elongated and colonized by *H. pylori* more frequently, compared with mice on the normal diet. Collectively, these studies in rodents support the hypothesis that salt can contribute to atrophy and function as a cocarcinogen.

To additionally investigate interactions between *H. pylori*, we designed an experiment where INS-GAS mice known to develop gastric cancer at 7 months after *H. felis* infection (21) were infected with *H. pylori* and fed a high-salt diet to determine whether infection and elevated dietary salt increased the severity of gastric lesions in these mice. We also included male and female mice in the study to determine whether gender influenced disease severity.

**MATERIALS AND METHODS**

**Animals.** Eighty-six (43 male and 43 female) weanling, specific pathogen-free INS-GAS mice on a FVB background (free of enteric *Helicobacter* spp, *Citrobacter rodentium*, *Salmonella* spp, endoparasites, ectoparasites, and serum antibodies to murine viral pathogens) obtained from an in-house breeding colony were used in the study (Table 1).

Animals were housed in microisolator, solid-bottomed polycarbonate cages and fed a commercially prepared pelleted diet and given water *ad libitum*. The mice were all maintained in an Association for Assessment and Accreditation of Laboratory Animal Care–approved facility under barrier conditions as virus antibody-free mice for the duration of the 7-month experiment. The protocol

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2 To whom requests for reprints should be addressed, at Division of Comparative Medicine, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Building 16, Room 825C, Cambridge, MA 02139. Phone: (617) 253-1757; Fax: (617) 252-1877; E-mail: jfox@mit.edu.

3 The abbreviations used are: MNNG, N-methyl-N-nitro-N-nitrosoguanidine; WPI, weeks postinfection; cfu, colony-forming unit.
was approved by the Animal Care Committee of the Massachusetts Institute of Technology.

**Bacteria.** *H. pylori* Sydney strain was used for oral inoculation as described previously (22). The organism was grown for 48 h at 37°C under microaerobic conditions on 5% lysed horse blood agar. The bacteria were harvested after 48 h of growth, resuspended in PBS, assessed by Gram’s stain and phase microscopy for purity, morphology, and motility and tested for urease, catalase, and oxidase activity.

**Experimental Infection.** Forty-eight (divided equally among female and male) INS-GAS mice were p.o. infected with 10⁸ cfu *H. pylori* Sydney strain in 0.3 ml of PBS given three times every other day. Thirty-eight control mice were dosed with PBS only. Half of the infected and half of the control mice were fed a high-salt diet (7.5 g/ml NaCl) and phase microscopy for purity, morphology, and motility and tested for urease, catalase, and oxidase activity.

**Quantitative Culture.** The weight of the gastric tissue was determined by subtracting the weight of the tube containing media from the weight after adding tissue. The tissue was homogenized using glass tissue grinders and the homogenate diluted 100- and 1000-fold in Brucella broth containing FCS. One hundred μl of each dilution was spread on selective medium: blood agar base no. 2 (Difco) supplemented with 5% horse blood (Remel) and 50 μg/ml amphotericin B, 100 μg/ml vancomycin, 3.3 μg/ml polymyxin B, 200 μg/ml bacitracin, and 10.7 μg/ml naladixic acid (Sigma Chemical Company, St. Louis, MO). Plates were incubated microaerobically at 37°C for 3–5 days. After verification of *H. pylori* by Gram’s stain, urease, catalase and oxidase reactions. Bacterial colonies were counted and the cfu/g of tissue calculated. Comparisons between groups were based on the log concentrations of bacteria.

**Histological Evaluation.** At necropsy, the stomach and proximal duodenum were removed and incised along the line of the greater curvature. Laminal contents were removed and the mucosa rinsed with sterile saline. For histopathology, linear strips extending from the squamocolumnar junction through the pyloric antrum were taken along the lesser curvature, placed on a card, and fixed in either 10% neutral-buffered formalin or Carnoy’s fixative. Tissues were routinely paraffin embedded, cut at 5 μm, and stained with H&E for histopathological evaluation.

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**Immunohistochemistry.** Mice received a single infection of BrdUrd at a dose of 5 mg/kg i.p. 1 h before euthanasia (20). Unstained, deparaffinized, Carnoy’s-fixed sections were stained for BrdUrd incorporation as described elsewhere (20). Additionally, formalin-fixed sections were antigen retrieved with pepsin (Zymed, San Francisco, CA) for 10 min at 37°C and were labeled with a polyclonal rabbit antibody recognizing the cleaved (activated) form of human caspase-3 (Cell Signaling Technologies, Beverly, MA) or rat monoclonal antibody recognizing mouse leukocyte common antigen (CD45, Ly-5; BD PharMingen, San Diego, CA). Primary antibody binding was detected with species-appropriate biotinylated secondary antibodies (Sigma Chemical Company), streptavidin peroxidase, and 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA), and tissues were counterstained with methyl green (Vector Laboratories). Immunohistochemical assays were performed on an automated immunostainer (i6000, Biogenex, San Ramon, CA).

**Quantitative Morphometry for Cell Proliferation.** BrdUrd-labeled cell numbers were compared between groups at the 7-month time point by morphometric analysis. Images of tissue sections stained for BrdUrd by immunohistochemistry (described above) were captured with a DXM1200 digital camera (Nikon Instruments, Melville, NY) and morphometric analysis was performed using IPLab 3.5 software for Macintosh (Scanalytics, Inc., Fairfax, VA). Three images of well-oriented oral gastric corpus/animal were analyzed. The gastric mucosa was manually outlined with an electronic drawing tablet (Graphire 2; Wacom Technology, Vancouver, WA), and the bounded area was defined as the region of interest. Color thresholding was used to selectively highlight labeled cells (brown, against a green background).

**Serology.** Evaluation of serum antibody responses to *H. pylori*. Serum was collected at 2, 4, 5, and 7 months and evaluated by ELISA for serum IgG2a (Th1-promoted isotype) and IgG1 (Th2-promoted isotype) using an outer membrane antigen preparation of *H. pylori* as described previously (24). Antigen was coated on plates at a concentration of 10 μg/ml, and sera were diluted 1:100. Biotinylated secondary antibodies included monoclonal anti-mouse antibodies produced by clones G1-6.5 and R19-157 (BD PharMingen) for detecting IgG1 and IgG2a, respectively. Incubation with extravidin peroxi-

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**Table 1** INS-GAS mice infected with *H. pylori* and fed basal or high-salt diet

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**Fig. 2.** Gastric histopathology demonstrating significantly more severe inflammation, hyperplasia, and dysplasia in *H. pylori*-infected male mice than females. A similar but less pronounced gender effect was observed in uninfected mice (data not shown). Basal versus high-salt diet had no significant effect on lesion scores. Samples from mice harvested at 5 months postinoculation. (a) male, basal diet; (b) male, basal diet; (c) female, high-salt diet; and (d) male, high-salt diet. H&E, original magnification: ×100.
idase (Sigma Chemical Company) was followed by ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for color development. Absorbance development at 405A was recorded by an ELISA plate reader (Dynatech MR7000; Dynatech Laboratories, Inc., Chantilly, VA).

**Statistical Analysis.** An ANOVA was performed to examine the effect of *Helicobacter* infection, gender, high-salt diet, and duration of infection (time) on the corpus and antral gastric lesions, *Helicobacter* colonization, and serological responses of INS-GAS transgenic mice. In addition to the level of colonization in each of the gastric compartments, there were five histopathological measures of disease assessed in the corpus; inflammation, glandular atrophy, hyperplasia, metaplasia, and dysplasia/neoplasia, and two antral measures; inflammation and hyperplasia that were assessed. The ANOVA model for each disease marker contained all independent variables and significant two-way interaction terms.

To investigate the influence of gender, diet and time on the pathogenesis of helicobacter-induced disease in more depth, a second ANOVA was performed for each lesion variable by the data from infected animals only. Graphs of the significant interactions were constructed to study the relationship of all interactions in greater detail. The colonization data were log transformed before any analyses. They were then subjected to analysis with the same ANOVA model as that used to analyze the histopathology scores. For BrdUrd analysis, percentages of the region of interest (i.e., gastric mucosa) containing BrdUrd label were tabulated and analyzed statistically using Prism 3.0 software for Macintosh (GraphPad, San Diego, CA). Mean differences between groups were assessed by one-way ANOVA, with values ≤ 0.05 considered significant. Comparisons between pairs of groups were performed using the Student t test.

**Gastrin Radioimmunoassay.** Plasma gastrin levels (COOH-terminally amidated gastrin) were determined by radioimmunoassay using rabbit antisera L2 that reacts similarly with G17 and G34 (23).

**RESULTS**

At the 5-month collection point, both infected and uninfected INS-GAS mice on either the basal or high-salt diet exhibited minimal-to-mild hyperplasia and dysplasia in the females and mild-to-moderate changes in the males. However, this gender effect was more pronounced in the infected mice (Fig. 1). Unlike gender, diet exerted no significant effect. Additionally, there was moderate loss of chief cells and mild loss of parietal cells with equivalent gender-specific severity among these groups. At the 7-month time point, uninfected INS-GAS mice demonstrated more severe hyperplasia, dysplasia, and chief and parietal cell atrophy than at the 5-month time point, with an equivalent gender effect (Fig. 2, a and c). Again, there was no appreciable difference between the uninfected mice fed the basal diet versus those given the high-salt diet. *H. pylori* infection significantly exacerbated disease in all groups (Fig. 2, b and d). Surprisingly, lesion scores in the mice on the basal diet were slightly higher than those on the high salt diet among infected mice. Lesions consistent with in situ and intramucosal carcinoma were only observed in male mice in the basal diet group. Four of 6 *H. pylori*-infected male mice on the basal diet, infected for 7 months, were diagnosed with gastric cancer, and all remaining infected male mice on both diets exhibited severe dysplasia and preneoplastic lesions (Fig. 3).

*H. pylori* infection in mice resulted in gastric mucosal inflammation, epithelial hyperplasia, metaplasia, and dysplasia (Fig. 4). Changes included elongated branched and tortuous glands, cystic...
dilation with crypt abscesses, and pseudostratification up to several cells deep. More severely affected animals exhibited multifocal intestinal metaplasia characterized by epithelial columnar elongation, microvillous brush borders, and single large cytoplasmic round vacuoles. Within dysplastic foci were single apoptotic cells characterized by diffusely granular to hyaline eosinophilic cytoplasm, karyorrhexis, and nuclear pyknosis. Phagocytosed apoptotic cells or their remnants were common in cytoplasmic vacuoles in neighboring epithelial cells. Transition zones from normal to hyperplastic to dysplastic epithelium were usually discernible and occurred in a wave of decreasing severity aborally from the cardia such that lesions were invariably most severe adjacent to the cardia. Moreover, there was severe chief cell atrophy that was complete in some males and losses up to 50–75% of parietal cells. In some mice, chief cell areas displayed a mucous neck cell phenotype, suggesting dedifferentiation to a more primordial state or absence of differentiation during downward migration. Parietal cells, sometimes in large numbers, exhibited small vacuolar to foamy metaplasia, taking on an appearance reminiscent of duodenal submucosal (Brunner’s) gland cells (Fig. 4).

Dysplastic changes supportive of a diagnosis of carcinoma in situ included intraglandular folding and papillary and ductular projections, moderate to marked cellular pleomorphism, cellular atypia, including signet ring forms, euchromatic nuclei with up to two to three mitotic figures/high power field, and occasional bizarre mitotic figures (Fig. 4). Two animals exhibited features of intramucosal carcinoma, with glandular invasion into the muscularis mucosae. Dysplastic glands abutted submucosal lymphatics (Fig. 4), but frank submucosal invasion was not demonstrated.

**Immunohistochemistry.** Leukocyte common antigen (CD45) labeling (Fig. 5, a and b) subjectively was directly proportional to inflammation scores assigned to H&E-stained sections. Likewise, BrdUrd labeling was proportional to mucosal epithelial hyperplasia scores (see below), and mitotically active cells were encountered both above and below the normal proliferative zone, with disorganization mirroring the overall level of mucosal dysplasia (Fig. 5, c and d). Cleaved (activated) caspase-3 was detected in surface (terminal) epithelial cells regardless of degree of dysplasia or cellular atypia (Fig. 5, e and f), suggesting that dysplastic cells in this model retained the functional cell signaling pathways necessary for terminal apoptosis. Marked cellular pleomorphism and variation in surface epithelial thickness precluded meaningful quantitative comparison of the number of caspase-3-positive cells in dysplastic stomach sections; however, the relatively uniform immunolabeling of surface epithelium corroborated the widespread finding of apoptotic cells in H&E-stained sections.
Quantitative Morphometry for Cell Proliferation. Mice infected with *H. pylori* had more BrdUrd-labeled cells (i.e., proliferation/unit area of gastric mucosa than did uninfected mice (Fig. 5). This difference in BrdUrd labeling between groups was highly significant statistically (*P* < 0.0001). The difference was significant in both sexes, although males (*P* = 0.0001) demonstrated larger differences between infected and uninfected cohorts than did females (*P* = 0.035). Stratified by gender and diet, there was a statistically significant difference (*P* < 0.05) in every group between uninfected and infected mice, except females on the basal diet (*P* = 0.14), probably because of the large SD in the infected females on the basal diet. However, there were no statistically significant differences in BrdUrd labeling between males versus females or basal versus high-salt diet groups between mice with the same infection or noninfection status. Thus, the only risk factor associated with statistically significantly increased numbers of BrdUrd+ nuclei (i.e., cell proliferation) in the gastric mucosa was *H. pylori* infection.

Serum Antibody Responses to *H. Pylori*. The Th1-associated serum IgG2a response to *H. pylori* increased from the time of experimental infection to necropsy at 5 or 7 months (Fig. 6a). At 2, 4, 5, and 7 months postinfection, the IgG2a responses were similar among all experimentally infected mice with no influence of gender (*P* > 0.10) or salt content of the diet (*P* > 0.27). In contrast, the Th2-associated serum IgG1 response to *H. pylori* was significant by 2 months postinfection (*P* < 0.05) but plateaued in most groups of experimentally infected mice through the time of necropsy (Fig. 6b). The IgG1 response at 7 months postinfection in infected male INS-GAS mice on the high-salt diet was significantly higher than the infected males on the low-salt diet or infected females on either salt diet (*P* < 0.012).

Statistical Analysis. A series of ANOVAs was performed to determine the significance of *H. pylori* infection in the development of gastric lesions. There was a highly significant main effect for helicobacter infection status for all fundic and antral lesion parameters (*P* < 0.0001), as well as significant interactions between infection status and diet for all of the fundic parameters (all *P* < 0.03) with the exception of intestinal metaplasia. In contrast, although serum gastrin was highly correlated with fundic inflammation, hyperplasia, glandular atrophy, metaplasia, and dysplasia (all *P* < 0.004), there was no effect on gastrin levels associated with infection status (*P* > 0.4). There was a main effect for diet and time (all *P* < 0.01), although not for gender (*P* > 0.4), on serum gastrin. This was a reflection of the significantly higher gastrin levels observed at the later time point and the fact that gastrin levels were generally lower in animals on the high salt diet (Fig. 7).

In subsequent ANOVAs in which the data were limited to that from infected animals, there was a highly significant main effect for time, diet, and gender (all *P* < 0.02) on all of the corpus lesion parameters scored (inflammation, atrophy, hyperplasia, metaplasia, and dysplasia/neoplasia). Gastric pathology was more severe in males, increased with time and, surprisingly, was milder in animals on the high-salt diet. In addition, gender interacted significantly with time (all *P* < 0.03), which prompted additional exploration into the nature of these interactions.

The significant two-way interactions are depicted graphically in Figs. 8 and 9. When infection status is included as a variable, there is a significant diet by infection interaction (Fig. 8). Interestingly, for all fundic parameters, the high-salt diet results in an increase in pathology in the uninfected animals and a marked decrease in the infected animals. When only infected animals are considered in the analyses, there is a significant time by gender interaction (Fig. 9). Although time is an important factor in the pathogenesis of disease for both males and females, females have a delayed response to helicobacter infection relative to the males, reflected in the lower lesion scores at the 5-month time point but then show a greater increase in the severity of disease over time, as indicated by the steeper slopes of the female curves.

**H. pylori** Colonization. The single factor that significantly influenced colonization in the corpus and the antrum was duration of infection (all *P* < 0.0001; Fig. 10). Colonization of both the corpus and antrum, which occurred in all infected mice, was comparable at the 5-month time point and colonization in both of these gastric compartments increased significantly within the 2-month time frame of the study regardless of gender or diet.
DISCUSSION

In this study, we used the hypergastrinemic INS-GAS mouse model to study the effect of H. pylori infection and a high-salt diet on gastric cancer progression. We were able to document H. pylori-associated gastric cancer in INS-GAS mice. This appears to be the first report of the induction of gastric cancer by H. pylori in any mouse model and thus confirms the carcinogenicity of this organism. Moreover, the development of gastric cancer over the 7-month observation period was seen only in INS-GAS H. pylori-infected male mice on the low-salt diet.

We previously demonstrated that elevations in gastrin-17 levels have also been associated with an increased risk for gastric glandular atrophy and cancer (21). Elevation in levels of G-17 in INS-GAS transgenic mice on a FVB background, which we also used in this study, resulted in the spontaneous development of atrophy and gastric cancer in uninfected mice over a period of 1–2 years and more rapid development of cancer (7 months) in H. felis-infected mice (21). Our current study demonstrates that H. pylori can likewise induce gastric cancer in male but not female INS-GAS mice within 7 months postinoculation that is independent of dietary salt intake. Dysplastic and neoplastic changes observed in this study, which mirror lesions described in H. felis-infected INS-GAS mice and which are features of H. pylori-associated adenocarcinoma in humans and gerbils, include epithelial dysplasia, papillary and ductular hyperplasia, cellular atypia, and bizarre mitotic figures (25–27). Signet ring forms seen in the H. pylori-infected mice with gastric tumors is also seen in H. pylori-associated gastric adenocarcinoma of humans but was not noted in H. felis INS-GAS gastric tumors (21). However, tumors in H. pylori-infected INS-GAS mice covered less gastric surface area than did tumors induced by H. felis infection (unpublished observations), and vascular invasion was documented in H. felis- but not H. pylori-infected INS-GAS mice by 7 months postinfection (21). This variation in disease severity may reflect a more robust adaptation of H. felis to the murine stomach or may be the result of inherent differences in bacterial pathogenicity between the two strains [although H. felis is CagA (−) and VacA (−)].

Our study also supports a role for H. pylori-mediated hyperproliferation in the induction of gastric cancer. Immunohistochemical markers of cellular proliferation (BrdUrd incorporation) and apoptosis (activated caspase-3) demonstrated significantly increased mitotic activity associated with H. pylori infection. Although by morphometric analysis, males demonstrated higher BrdUrd labeling indices than did females, these differences did not reach statistical significance, probably because of individual biological and histological sampling variation. Subjective interpretation of caspase-3 labeling revealed no differences in the ability of epithelial cells to undergo apoptosis, regardless of hyperplasia or dysplasia status. Taken together, these findings suggest that the gastric epithelial layer thickening associated with H. pylori infection is attributable more to increased cell proliferation than to decreased apoptosis. Peek et al. (28) have shown that H. pylori infection in gerbils leads to increases in gastric epithelial proliferation that appear to be temporally related to increased serum gastrin levels. This group concluded that the epithelial cell growth in H. pylori-colonized mucosa of the Mongolian gerbil may be mediated...
by gastrin-dependent mechanisms. Thus, the two animal models described to date that appear most susceptible to Helicobacter-dependent gastric cancer—the INS-GAS mouse and the Mongolian gerbil—are both characterized by markedly elevated levels of amidated gastrin. In addition, at least one study has shown that *H. pylori*-infected patients elevated levels of amidated gastrin at baseline are more likely to progress to glandular atrophy after treatment with proton pump inhibitors (29).

In our study, gastric cancer was observed only in the infected male INS-GAS mice. Previous studies have shown that susceptibility to *Helicobacter*-mediated atrophy and preneoplasia is strongly influenced by genetics, but little attention has been paid to gender although male humans are at increased risk to develop gastric cancer. For example, several groups have demonstrated that C57BL mice have a vigorous Th1-mediated gastric inflammation after infection with *Helicobacter* spp, whereas the BALB/c mouse has minimal gastric inflammation (indicative of a Th2 response). In general, there is an inverse relationship between the strength of the Th1 response and the level of *Helicobacter* colonization (30). The cell-mediated immune hyporesponsiveness to gastric helicobacter infection observed in disease-resistant strains such as BALB/c and CBA mice appears to be a dominant trait, as evidenced by results from analysis of the gastric phenotype in F1 generation of CBA/c x C57BL infected with gastric helicobacters (31, 32). These investigators had also used both females and males but compared with our 7 month study, their study period was for 3 months, and a gender effect was not noted (31, 32). Indeed, many studies use female mice based on the practicality that cohoused female mice are less likely to fight when compared with cohoused male mice.

Recently, however, a gender effect in mice was described in an oral vaccination trial using interleukin receptor 4α chain-deficient BALB/c mice in which *H. pylori* native urease (5 μg) and cholera toxin (10 μg) administered at weekly intervals for 4 weeks provided protection against 10⁹ cfu of mouse-adapted P76 *H. pylori* (33). These authors noted higher colonization rates in *H. pylori*-infected control male interleukin receptor 4α mice and wild-type male BALB/c mice than in their female counterparts (33). This finding was statistically significant in interleukin receptor 4α−/− mice. Unfortunately, histopathological correlates of gastric disease between male and female mice were not presented (33). Our study documents clearly the greater susceptibility of male gastric tissue to *H. pylori* infection, a finding consistent with the higher rates of gastric carcinoma in men. The INS-GAS mouse model will likely be useful in additional investigations that will address the mechanisms responsible for this gender difference in susceptibility.

The most surprising finding was perhaps the absence of synergism in the INS-GAS mouse model between *H. pylori* infection and a high-salt diet. In an earlier study performed in *H. pylori*-infected C57BL/6 mice, we demonstrated that a high-salt diet accelerated the development of hyperproliferation, pit cell hyperplasia, and glandular atrophy (20). However, this C57/BL mouse study was terminated at 16 WPI, rather than the 7-month observation period in the current study (20). Nevertheless, we speculated that chronic salt intake may exacerbate gastritis by increasing *H. pylori* colonization density and theorized that the increased colonization might be attributable to several mechanisms (20). Terminal serum gastrin concentrations were statistically increased in *H. pylori*-infected C57BL mice on a normal diet versus uninfected controls (51.8 versus 23.7 pm) and elevated in
infected mice fed high-salt diets when compared with control mice on the same diet (20), although the serum gastrin concentrations did not wholly parallel the colonization data. Previous reports had suggested that high-salt diets can be associated with hypergastrinemia. Taken together, our results raised the possibilities that the observed salt-associated effects occurred in part through the induction of gastrin (20).

In this study, we noted an increase in H. pylori colonization density in INS-GAS mice over time that was independent of salt intake or gender, and we found that the high-salt diet showed a clear-cut protective effect in this animal model. Only the infected INS-GAS male mice on a low-salt diet developed gastric cancer. Overall, the INS-GAS/H. pylori mice on a high-salt diet showed decreased histological progression to atrophy and neoplasia, which was also reflected by lesser increases in serum gastrin levels, suggesting better preservation of acid secretion and parietal cell function. Possible explanations for these different findings include differences in murine genetic background in the two studies and the unique characteristics of the INS-GAS mice. Although the possibility cannot be excluded that FVB/N mice respond differently than C57BL/6 mice to a high-salt diet, a more plausible explanation relates to the robust nature of the INS-GAS model. It is likely that the unique and rapid progression to atrophy, metaplasia, and cancer seen in H. pylori-infected INS-GAS mice has eclipsed the procarcinogenic effect of the high-salt diet, thereby unmasking protective effects that would have otherwise been obscured. The absence of a additive effect by high salt in wild-type mice, e.g., C57BL mice, is acting, in part, through induction of hypergastrinemia (20). In INS-GAS mice, however, there is already a sufficient elevation of circulating gastrin such that high salt no longer has a potentiating effect. In addition, it is likely that high salt is less able to induce gastrin release in these mice because antral gastrin expression is highly suppressed by circulating pancreatic gastrin in the INS-GAS transgenic model. Thus, the absence of a potentiating effect by high salt in the INS-GAS model is most consistent with a model in which gastrin is a downstream target of high salt.

The mechanism by which high salt exerts its protective effect in the INS-GAS/H. pylori model is currently unknown. However, it is of interest that the high-salt diet in infected male mice was associated with an enhanced anti-inflammatory Th2 humoral immune response to H. pylori antigens, which correlated with less severe gastric lesions and the absence of clear neoplastic transformation at 7 months postinfection. Mouse strains exhibiting weak type-1 responses to H. pylori exhibited a significant Th2 cytokine response to helicobacter antigens, and these findings support our observation of an enhanced Th2 humoral immune response in the infected males fed a high-salt diet for 7 months postinfection. The possibility that high salt is able to modulate the immune and gastric cytokine response (e.g., interleukin 1β) to H. pylori no doubt bears additional investigation.

In summary, we did not see, as expected, a synergistic effect with salt and H. pylori in the INS-GAS mouse. This supports the notion that in the mouse model, constitutive hypergastrinemia and high dietary salt intake are nonadditive risk factors for development of H.
pylori-induced adenocarcinoma. This result is supportive of the model that gastrin acts downstream of and mediates the effects of high salt in the acceleration of gastric cancer, but additional studies are needed to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue. However, to our knowledge, this is the first study to clarify this issue.

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Helicobacter Pylori-associated Gastric Cancer in INS-GAS Mice Is Gender Specific

James G. Fox, Arlin B. Rogers, Melanie Ihrig, et al.


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