Mutagenic potency of Helicobacter pylori in the gastric mucosa of mice determined by sex and duration of infection

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th>Citation</th>
<th>Sheh, A. et al. “Mutagenic potency of Helicobacter pylori in the gastric mucosa of mice is determined by sex and duration of infection.” Proceedings of the National Academy of Sciences 107 (2010): 15217-15222 ©2011 by the National Academy of Sciences.</th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="http://dx.doi.org/10.1073/pnas.1009017107">http://dx.doi.org/10.1073/pnas.1009017107</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>National Academy of Sciences (U.S.)</td>
</tr>
<tr>
<td>Version</td>
<td>Final published version</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/66141">http://hdl.handle.net/1721.1/66141</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Article is made available in accordance with the publisher's policy and may be subject to US copyright law. Please refer to the publisher's site for terms of use.</td>
</tr>
<tr>
<td>Detailed Terms</td>
<td></td>
</tr>
</tbody>
</table>
Mutagenic potency of *Helicobacter pylori* in the gastric mucosa of mice is determined by sex and duration of infection

Alexander Sheh\(^b\), Chung Wei Lee\(^a,b\), Kenichi Masumura\(^c\), Barry H. Rickman\(^b\), Takehiko Nohmi\(^d\), Gerald N. Wogan\(^a,1\), James G. Fox\(^a,b,2\), and David B. Schauer\(^a,b,2\)

\(^a\)Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139; \(^b\)Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 02139; and \(^c\)Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo 158-8501, Japan

Contributed by Gerald N. Wogan, July 19, 2010 (sent for review April 27, 2010)

*Helicobacter pylori* is a human carcinogen, but the mechanisms evoked in carcinogenesis during this chronic inflammatory disease remain incompletely characterized. We determined whether chronic *H. pylori* infection induced mutations in the gastric mucosa of male and female gpt delta C57BL/6 mice infected for 6 or 12 mo. Point mutations were increased in females infected for 12 mo. The mutation frequency in this group was 1.6-fold higher than in uninfected mice of both sexes (*P* < 0.05). A:T-to-G:C transitions and G:C-to-T:A transversions were 3.8 and 2.0 times, respectively, more frequent in this group than in controls. Both mutations are consistent with DNA damage induced by oxidative stress. No increase in the frequency of deletions was observed. Females had more severe gastric lesions than males at 6 mo postinfection (MPI; *P* < 0.05), but this difference was absent at 12 MPI. In all mice, infection significantly increased expression of *Ifnγ*, *Il-17*, *Tnfα*, and *iNos* at 6 and 12 mo, as well as *H. pylori*-specific IgG1 levels at 12 MPI (*P* < 0.05) and IgG2c levels at 6 and 12 MPI (*P* < 0.01 and *P* < 0.001). At 12 MPI, IgG2c levels in infected females were higher than at 6 MPI (*P* < 0.05) and also than those in infected males at 12 MPI (*P* < 0.05). Intensity of responses was mediated by sex and duration of infection. Lower *H. pylori* colonization indicated a more robust host response in females than in males. Earlier onset of severe gastric lesions and proinflammatory, Th1-biased responses in female C57BL/6 mice may have promoted mutagenesis by exposing the stomach to prolonged oxidative stress.

\textit{gpt} delta mouse | *Helicobacter* | inflammation | mutagenesis | sexual dimorphism

\section*{Results}

**Mutagenicity and Mutation Spectrum.** DNA from the gastric mucosa of infected p53\(^{-}\) and uninfected p53\(^{+/+}\) mice was recovered from the lacI-gpt reporter gene and analyzed by SSCP and sequencing to confirm the presence of point mutations. At 6 and 12 mo post infection, the frequency of point mutations at 7 MPI, compared with uninfected controls (23), was significantly increased in females but not in males. The frequency of point mutations was higher in females infected with *H. pylori* than in wild-type (WT) mice infected with *H. pylori* (22). Both female and male infected mice of both sexes (22) and infected with *H. pylori* developed robust gastritis (23). Female mice infected with *H. felis* also had an increased intensity of point mutations at 7 MPI, compared with uninfected controls (23). Mutagenesis resulting from infection has also been associated with p53 status. Mutaions were found in the lacI reporter genes of a small number of *H. felis*-infected female TSG-p53\(^{-}\)Big Blue mice harboring either one (p53\(^{+/−}\)) or two (p53\(^{+/−}\)) WT p53 alleles (23). A 2-fold increase in point mutations was found in DNA from the gastric mucosa of infected p53\(^{+/−}\) mice, and also in uninfected p53\(^{+/−}\) mice; the mutation frequency in infected p53\(^{+/−}\) mice was further increased by approximately threefold. The intensity of inflammation was estimated to be significantly higher in infected p53\(^{+/−}\) mice than in infected p53\(^{+/+}\) animals, and gastric epithelial proliferation was similarly increased with infection in both latter treatment groups. By contrast, in another study, infection of Big Blue transgenic mice (sex not specified) with the SS1 strain of *H. pylori* for 3.5 mo resulted in no significant increase in gastric mutations over uninfected controls (13).

We used the gpt delta mouse to measure the accumulation of genetic mutations associated with *H. pylori* SS1 infection in male and female animals at 6 and 12 MPI. This experimental system comprises λ-EG10–based transgenic C57BL/6 mice harboring tandem arrays of 80 copies of the bacterial gpt gene at a single site on chromosome 17. The model was specifically designed to facilitate the in vivo detection of point mutations by 6-thioguanine.
(6-TG) selection, and deletions up to 10 kb in length by selection based on sensitivity to P2 interference (Spi+) (24). When phage DNA rescued from mouse tissues is introduced into appropriate Escherichia coli strains, both point mutations and large deletions can be efficiently detected (24). We also characterized histopathologic changes, expression of inflammatory cytokines, and expression of iNOS in gastric mucosa 6 and 12 mo after initial infection. In addition, we compared responses of male and female mice to assess the influence of sex. We found that H. pylori infection induced significant increases in the frequency of point mutations in the gastric mucosa of female, but not male, gpt delta mice. The accumulation of point mutations was therefore sex-dependent and was mediated by the duration of infection and the severity of disease.

Results and Discussion

Pathology, Cytokine and iNOS Expression, and Serologic Responses to H. pylori Infection and H. pylori Levels. Inflammatory histopathologic changes resulting from H. pylori infection were evaluated (Fig. S1) and gastric histological index (GHAI) scores calculated, with the following results. Scores were significantly increased in infected animals of both sexes at 6 and 12 MPI (female, P < 0.001 and P < 0.01, respectively; and male, P < 0.05 and P < 0.001, respectively; Fig. 1). Regarding specific types of lesions represented in the GHAI, infected females experienced higher levels of hyperplasia (P < 0.05), epithelial defects (P < 0.001), and dysplasia (P < 0.05) compared with infected males at 6 MPI, and thus had correspondingly higher (P < 0.05) GHAI scores at this time point. At 6 MPI, infected females (female, P < 0.001 and P < 0.01) demonstrated mild to moderate inflammation, comprised chiefly of submucosal and mucosal infiltrates of mononuclear and granulocytic cells. In addition, in infected males, mucous metaplasia, intestinal metaplasia, oxyntic gland atrophy, and hyalinosis were all significantly elevated compared with controls (all P < 0.01; intestinal metaplasia, P < 0.001); no significant increases occurred in foveolar hyperplasia, epithelial defects, or dysplasia. In infected females, inflammation (P < 0.05), epithelial defects (P < 0.001), mucous metaplasia (P < 0.01), hyalinosis (P < 0.001), as well as premalignant lesions of oxyntic gland atrophy (P < 0.001), intestinal metaplasia (P < 0.05), and dysplasia (P < 0.05) were significantly elevated compared with uninfected controls. At 12 MPI, there were no differences in gastric lesion severity between infected males and females; both groups had similar mucosal changes. Infected male mice at 12 MPI displayed significantly increased epithelial defects but decreased intestinal metaplasia than infected males at 6 MPI (P < 0.01 and P < 0.001).

H. pylori infection significantly increased transcription profiles at 6 and 12 MPI as follows (details in Fig. S2): IFN-γ (males, P < 0.001; females, P < 0.01 at 6 and 12 MPI); TNF-α (males, P < 0.01 at 6 and 12 MPI; females, P < 0.01 and P < 0.05); IL-17 (males, P < 0.05 and P < 0.001; females, P < 0.05 and P < 0.01). iNOS expression was increased in male and female mice at 6 and 12 MPI (males, P < 0.05 and P < 0.01; females, P < 0.01 and P < 0.001) compared with controls at 12 mo. IL-10 expression was not significantly affected by infection in animals of either sex (P > 0.05).

H. pylori infection also resulted in a Th1-predominant IgG2c response in infected mice as previously reported (25, 26) (Fig. 2). H. pylori-specific IgG2c levels were higher in infected than in uninfected animals at 6 and 12 MPI (P < 0.01 and P < 0.001, respectively). At 12 MPI, infected females had significantly higher

![Fig. 1](https://www.pnas.org/cgi/doi/10.1073/pnas.1009017107)

Fig. 1. H. pylori infection elicits more gastric pathologic processes in female mice at 6 mo. H. pylori infection increased the GHAI in both male and female C57BL/6 mice at 6 and 12 mo. At 6 mo of infection, infected females had significantly more pathologic processes than infected males. Uninfected males (♀; 6 MPI, n = 7; 12 MPI, n = 3), uninfected females (♀; 6 MPI, n = 5; 12 MPI, n = 3), infected males (♂; 6 MPI, n = 4; 12 MPI, n = 5) and infected females (♀; 6 MPI, n = 4; 12 MPI, n = 6). Bar represents the mean. *P < 0.05, **P < 0.01, and ***P < 0.001.

![Fig. 2](https://www.pnas.org/cgi/doi/10.1073/pnas.1009017107)

Fig. 2. The effect of H. pylori infection on H. pylori-specific IgG1 and IgG2c. Serum levels of IgG were measured by ELISA in uninfected and H. pylori–infected mice. (A) H. pylori–infected mice (gray bars; 6 MPI, n = 9; 12 MPI, n = 12) developed a greater IgG1 response after 12 mo of infection compared with uninfected mice (white bars; 6 MPI, n = 6; 12 MPI, n = 6; P < 0.05). (B) At 12 mo, infected female mice (light gray bars; 6 MPI, n = 4; 12 MPI, n = 6) had substantially increased IgG2c compared with infected males (dark gray bars; 6 MPI, n = 5; 12 MPI, n = 6) at 12 mo and infected females at 6 mo (P < 0.05, both). Data are mean (SE) of mice in different treatment groups. *P < 0.05, **P < 0.01, and ***P < 0.001.
IgG2c levels than infected males (P < 0.05). Duration of infection also increased IgG2c levels in infected females, with higher levels noted at 12 versus 6 MPI (P < 0.05). H. pylori-specific IgG1 (Th2) levels were higher in infected than in uninfected animals at 12 MPI (P < 0.05). Females had higher Th1/Th2 ratios than males; at 6 MPI, 8.58 vs. 4.01; and at 12 MPI, 12.9 vs. 7.51.

We and others have demonstrated that H. pylori levels are inversely correlated with the degree of pathology and the immune response as measured by antibody and cytokine production (25, 27). At 6 MPI, H. pylori was detectable by quantitative PCR in all infected males, but not in one female. At 12 MPI, there was a salient difference in levels of H. pylori colonization between males and females; H. pylori was undetectable in four infected females, but undetectable in only one male (Fig. 3).

**Frequency and Nature of Mutations.** We determined the frequency of gpt point mutations in gastric DNA isolated from infected and uninfected mice by selection of mutants based on 6-TG resistance. Recognizing the possible confounding effect of clonal expansion of sibling mutants (i.e., jackpot mutations), we sequenced the gpt genes from all 566 recovered mutants. Any mutation found to duplicate another at the same site within an individual sample was excluded from subsequent frequency calculations. After this adjustment, at 12 MPI the gpt mutation frequency in infected females (7.5 ± 2.0 × 10^-6; P < 0.05) was significantly (1.6-fold) higher than that in all control animals (4.7 ± 1.1 × 10^-6), whereas the frequency in infected males (6.3 ± 5.4 × 10^-6; P = 0.49) was not.

At 6 MPI, gpt mutation frequency in infected animals of either sex, females, 5.6 ± 2.0 × 10^-6; P = 0.72; males, 7.2 ± 2.2 × 10^-6, P = 0.57) was not significantly different from that of controls (6.2 ± 3.0 × 10^-6; Fig. 4).

After sequencing and accounting for clonal expansion, 236 from infected and 156 mutants from uninfected mice were used to identify effects of H. pylori infection on types of mutations causing loss of gpt function, with the results summarized in Table 1 and Table S1. G:C-to-A:T transitions and G:C-to-T:A transversions were the most prevalent types of mutations in both infected and uninfected animals, representing 33% to 50% and 17% to 31%, respectively, of total mutations in various treatment groups; mutant frequencies varied from 2.1 to 3.0 × 10^-6 in these transitions and 0.9–1.8 × 10^-6 in the transversions. The 1.6-fold higher total mutations observed in infected females compared with age-matched controls at 12 MPI was attributable mainly to two types of mutations: 3.8 times more A:T-to-G:C transitions (P < 0.05) and 2.0 times more G:C-to-T:A transversions (P < 0.05). These two mutations are consistent with the expected mutational spectrum induced by RONS (28) and have been found elevated in another animal model of chronic inflammation (13). An increase in G:C-to-T:A transversions was also reported in a previous study documenting H. pylori-induced mutations (22).

A:T-to-G:C transitions can be formed by deamination of adenine to hypoxanthine or creation of ethenoadenine. Deamination is mediated by N_{2}O_{3}, the autoxidation product of NO_{x}, which directly nitrosates primary amines on DNA bases (28, 29). Hypoxanthine resembles guanine and mispairs with cytosine, resulting in the observed mutation. Alternatively, A:T-to-G:C transitions can also be created indirectly by lipid peroxidation by RONS, forming etheno adducts in DNA such as highly mutagenic ethenoadenine (30, 31). G:C-to-T:A transversions have also been associated with increased iNOS expression levels, pointing to the involvement of RONS (32). Cells cocultivated with activated macrophages predominantly develop G:C to T:A transversions caused by exposure to NO\textsubscript{2}, O_{2}^{-}, and H_{2}O_{2} (33). G:C-to-T:A transversions are also caused by adducts produced by oxidative stress or lipid peroxidation, such as 8-oxoG, dC, and M1G (34, 35). The presence of 8-oxoG, a major product of oxidative damage to DNA, results in mispairing of guanine with adenine during replication, thus inducing transversions (36). Although 8-oxoG is believed to be the main cause of this mutation, etheno adducts formed by lipid peroxidation may also play a significant role in mutagenesis observed in vivo (37).

**Fig. S3** shows the mutation spectrum detected in the gpt gene, of which the following features are noteworthy. In both uninfected and infected mice, hotspots occurred at nucleotides C64, G110, G115, and G418; C64, G110, and G115 are CpG sites and are known hotspots in this assay (38, 39). Hotspots occurring in infected mice were located at A8, G116, and G143; A8 and G116 were found predominantly in infected females at both 6 and 12 mo, whereas G143, located at a CpG site, was a hotspot in infected animals of both sexes. Uninfected mice had hotspots at G406, G416, and A419, one of which (G416) occurred mainly in males. Mutations in infected males were concentrated mainly in hotspots common to both uninfected and infected animals, whereas in infected females there was an increase in mutations throughout the gpt gene at non-G-C sites. This result suggests that the chemistry of DNA damage in mice with a stronger host response to infection may have differed from that in mice with mild or minimal gastritis.
assay revealed that the frequency of gastric tissue DNA was not increased in infected males at 12 MPI, compared with infected females, whereas by 12 MPI, the level of pathologic process was similar in both sexes. These data suggest that the delayed onset of severe gastric lesions in males reduced the duration of their exposure to chronic gastritis, protecting them from mutagenesis, highlighting the importance of severity and duration of the inflammatory response.

Our findings agree with current paradigms for the role of inflammation in carcinogenesis (4) and with the more severe pathology induced in female versus male C57BL/6 mice infected with Helicobacter spp. (42). Our observations in female gpt delta mice are consistent with previously reported data from female C57BL/6 Big Blue mice infected with H. felis, which induces more severe gastritis at earlier time points than H. pylori, effectively increasing the amount of DNA damage inflicted after infection (23, 43, 44). The higher mutation frequency found at 7 MPI in H. felis-infected female C57BL/6 Big Blue mice may be comparable to that occurring at 12 MPI in female gpt delta mice infected with H. pylori, based on increased inflammation and epithelial proliferation. In contrast, another study of DNA point mutations induced by H. pylori in male Big Blue mice reported that, although mutant frequency was increased at 6 MPI, it returned to control levels by 12 MPI (22). The decrease between 6 and 12 MPI was accompanied by loss of iNOS expression and reversion of the mutation spectrum to one indistinguishable from that of uninfected mice (22). Two possible explanations for these results are that (i) the increase in mutations at 6 MPI may have reflected a temporary increase in the inflammation or (ii) loss of infection resulted in absence of iNOS expression and reversion of the mutation spectrum. An additional factor pertinent to comparisons of previous mutagenesis studies involving gastric infection by Helicobacter spp. is the endogenous Helicobacter status of the mouse colony (45). We recently reported that concurrent, subclinical infection in C57BL/6 mice with non-gastric Helicobacter bilis significantly reduced H. pylori-associated premalignant gastric lesions at 6 and 11 MPI (46). This immunomodulatory effect could affect observed mutagenic responses.

Table 1. Twelve months of H. pylori infection increases the mutation frequency of A:T-to-G:C transitions and G:C-to-T:A transversions in female mice

<table>
<thead>
<tr>
<th>Effect</th>
<th>Uninfected all (n = 6)</th>
<th>Infected male (n = 4)</th>
<th>Infected female (n = 8)</th>
<th>Uninfected all (n = 14)</th>
<th>Infected male (n = 5)</th>
<th>Infected female (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G:C to A:T</td>
<td>3.02 (2.07)</td>
<td>2.41 (2.64)</td>
<td>2.74 (1.07)</td>
<td>2.13 (0.87)</td>
<td>2.66 (1.70)</td>
<td>2.69 (0.97)</td>
</tr>
<tr>
<td>A:T to G:C</td>
<td>0.27 (0.61)</td>
<td>0.00</td>
<td>0.16 (0.22)</td>
<td>0.23 (0.19)</td>
<td>0.08 (0.16)</td>
<td>0.88 (0.59)*</td>
</tr>
<tr>
<td>Transversion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G:C to T:A</td>
<td>1.30 (1.34)</td>
<td>1.64 (1.29)</td>
<td>0.89 (0.68)</td>
<td>0.85 (0.62)</td>
<td>1.69 (2.43)</td>
<td>1.75 (0.54)*</td>
</tr>
<tr>
<td>G:C to C</td>
<td>0.29 (0.66)</td>
<td>0.36 (0.71)</td>
<td>0.40 (0.60)</td>
<td>0.19 (0.27)</td>
<td>0.84 (1.25)</td>
<td>0.09 (0.14)</td>
</tr>
<tr>
<td>A:T to T:A</td>
<td>0.34 (0.47)</td>
<td>0.00</td>
<td>0.37 (0.37)</td>
<td>0.31 (0.41)</td>
<td>0.00</td>
<td>0.53 (0.89)</td>
</tr>
<tr>
<td>A:T to G</td>
<td>0.00</td>
<td>0.36 (0.71)</td>
<td>0.18 (0.22)</td>
<td>0.05 (0.12)</td>
<td>0.00</td>
<td>0.25 (0.46)</td>
</tr>
<tr>
<td>Deletion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-bp deletion</td>
<td>0.84 (0.94)</td>
<td>2.44 (3.99)</td>
<td>0.66 (0.43)</td>
<td>0.53 (0.35)</td>
<td>0.67 (0.54)</td>
<td>0.88 (0.73)</td>
</tr>
<tr>
<td>≥2-bp deletion</td>
<td>0.04 (0.12)</td>
<td>0.00</td>
<td>0.44 (0.57)</td>
<td>0.22 (0.20)</td>
<td>0.18 (0.36)</td>
<td>0.22 (0.34)</td>
</tr>
<tr>
<td>Insertion</td>
<td>0.00</td>
<td>0.00</td>
<td>0.08 (0.16)</td>
<td>0.20 (0.23)</td>
<td>0.00</td>
<td>0.11 (0.17)</td>
</tr>
<tr>
<td>Complex mutation</td>
<td>0.14 (0.31)</td>
<td>0.00</td>
<td>0.04 (0.08)</td>
<td>0.00</td>
<td>0.18 (0.36)</td>
<td>0.12 (0.29)</td>
</tr>
<tr>
<td>Total</td>
<td>6.23 (2.98)</td>
<td>7.20 (2.21)</td>
<td>5.95 (1.54)</td>
<td>4.71 (1.13)</td>
<td>6.29 (5.37)</td>
<td>7.51 (1.91)*</td>
</tr>
</tbody>
</table>

Data are mean (SD) of mutation frequency data from 411 mutants recovered from the gpt assay after excluding 155 mutants considered siblings. Control mice of both sexes were grouped for this analysis. The mutation frequency of A:T-to-G:C transitions and G:C-to-T:A transversions was significantly elevated in female mice infected with H. pylori for 12 mo. *P < 0.05.

Mutation analysis using the Spi assay revealed that the frequency of deletion mutations in gpt of gastric tissue DNA was not significantly affected by H. pylori infection (Fig. 5). Analyses of 57 samples from 32 mice showed that Spi mutant frequencies in infected mice (females at 6 MPI, 4.8 ± 2.5 × 10⁻⁶; P = 0.09; females at 12 MPI, 5.0 ± 2.6 × 10⁻⁶; P = 0.30; males at 6 MPI, 6.3 ± 2.5 × 10⁻⁶; P = 0.87; and males at 12 MPI, 5.2 ± 1.4 × 10⁻⁶; P = 0.17) were not significantly different from those of age-matched controls at either time point (all mice at 6 MPI, 2.9 ± 1.3 × 10⁻⁶; all mice at 12 MPI, 3.6 ± 1.8 × 10⁻⁶).

Current models of inflammation-driven carcinogenesis are based on chronic inflammation inducing mutations that lead to cancer (40, 41). Female mice at 6 MPI had more hyperplasia, epithelial defects, and dysplasia than infected males and age-matched controls, but this increase in pathologic findings was not accompanied by increased frequency of mutations. This was detected only in infected female mice at 12 MPI, which had experienced more severe gastritis for a longer period, suggesting that gastritis is necessary but not sufficient to induce mutagenesis. Similarly, duration of infection in itself was insufficient, as mutation frequency was not increased in male mice at 12 MPI. Based on the GHAI, infected male mice had a weaker response at 6 MPI compared with infected females, whereas by 12 MPI, the level of pathologic process was similar in both sexes. These data suggest that the delayed onset of severe gastric lesions in males reduced the duration of their exposure to chronic gastritis, protecting them from mutagenesis, highlighting the importance of severity and duration of the inflammatory response.

Fig. 5. Mutant frequency of deletions was unchanged by H. pylori infection. H. pylori infection did not alter the levels of deletions detected by the Spi assay in uninfected mice (white bars; 6 MPI, n = 12; 12 MPI, n = 16), H. pylori-infected males (light gray bars; 6 MPI, n = 3; 12 MPI, n = 5) and H. pylori-infected females (dark gray bars; 6 MPI, n = 9; 12 MPI, n = 12). Control mice of both sexes were grouped for this analysis. Data are mean (SEM) of mutant frequency of mice in different treatment groups.
and it is unknown whether Big Blue mice used in previous studies were free of enteric Helicobacter spp.

Our observed sex-based effects on mutagenesis induced by *H. pylori* in mice has not been reported previously, but sex bias has been found in other responses to infection. In *H. pylori*-infected Mongolian gerbils, immune responses and cytokine production were reported to be affected by sex (47), and *H. pylori* preferentially induced cancer in male INS-GAS mice (48). Greater female susceptibility to gastric Helicobacter infections has been noted previously in WT C57BL/6 mice (42), whereby females infected with *H. felis* experience an earlier onset of gastric inflammation, epithelial hyperplasia, atrophy, and apoptosis (42). Mechanisms responsible for the observed sex-based effects are incompletely understood, but findings to date collectively indicate that sex is an important variable, affecting strength of the host response to *H. pylori* infection, which in turn determines disease outcome.

The typical host response to *Helicobacter* infection is a proinflammatory Th1 response that causes chronic gastritis (49, 50). However, mouse strains such as BALB/c, which mount a strong antiinflammatory Th2 humoral immune response to *H. pylori* infection, develop less severe disease (51). We have previously shown that modulation of the Th1 response by an increased Th2 response reduces pathology associated with concurrent helminth infections (27). In the current study, the immune response of females to *H. pylori* infection was biased toward a greater Th1/Th2 ratio compared with males at both 6 and 12 mo. Higher Th1/Th2 ratios reflect a stronger inflammatory response to *H. pylori* infection. Furthermore, proinflammatory Th17 cells and regulatory T cells have been recently shown to modulate host responses to *H. pylori* (52, 53). *H. pylori*-specific Th17 immunity, mediated by IL-17 (54), increases inflammation unless it is suppressed by regulatory T cells, which are up-regulated by TGF-β and IL-10 (55). Higher levels of inflammation, epithelial defects, and atrophy were indeed observed in infected females at 6 MPI. At 12 MPI, the level of serum IgG2c was significantly elevated in infected females, reflective of increased proinflammatory cytokines in the gastric mucosa, and is consistent with the reduction in *H. pylori* colonization levels. The earlier onset of severe pathologic process caused by the Th1- and Th17-biased response to *H. pylori* was also associated with the increase in point mutations seen at 12 MPI.

In summary, we have shown that chronic *H. pylori* infection can cause premalignant gastric lesions and induce point mutations consistent with inflammatory processes. As our data are derived from analysis of nontranscribed DNA, it serves as an indicator of unbiased mutations reflecting genetic changes during the early stages of tumor initiation in inflamed tissues. At 12 mo, *H. pylori*-infected female C57BL/6 mice accumulate more inflammation-mediated point mutations compared with males as a result of a greater Th1-biased response to infection inducing earlier and more severe pathology. The sex-biased increase in premalignant gastric lesions and induction of mutations highlights the importance of taking into account sex-based effects in future studies of inflammation-driven disease.

**Materials and Methods**

**Bacteria and Animals.** *H. pylori* strain S51 was grown on blood agar or Brucella broth with 5% FBS as described in SI Materials and Methods. Specific pathogen-free (including *Helicobacter* spp.) male and female C57BL/6 gpt delta mice (24) were infected by oral gavage with *H. pylori* S51 or sham-dosed. At the indicated times, mice were euthanized, gastric tissue collected for histopathology and DNA and RNA extraction, and sera were collected for cytokine and Ig analysis. Gastric lesions were scored for inflammation, epithelial defects, atrophy, hyperplasia, mucous metaplasia, hyalinosis, intestinal metaplasia, and dysplasia using previously published criteria (55). The GHA1 is the sum of inflammation, epithelial defects, atrophy, hyperplasia, intestinal metaplasia, and dysplasia scores. A detailed description of the histroyan, treatment, and histopathology is provided in SI Materials and Methods.

**DNA Isolation and in Vitro Packaging.** Genomic DNA was extracted from gastric tissue using RecoverEase DNA Isolation Kit (Stratagene) following the manufacturer’s recommendations. E. coli HY6620 expressing Cre recombinase. Infected cells were cultured on plates containing chloramphenicol (Cm) and 6-TG for 3 d until 6-TG-resistant colonies appeared. To confirm the 6-TG-resistant phenotype, colonies were restreaked on plates containing Cm and 6-TG. Confirmed 6-TG-resistant colonies were cultured, and a 739-bp DNA product containing the gpt gene was amplified by PCR. DNA sequencing of the gpt gene was performed by the Biopolymers Facility at Harvard Medical School (Boston, MA) with Amplicon markers (Agencourt) and a 3730XL DNA Analyzer (Applied Biosystems). Sequences were aligned with the *E. coli* gpt gene (GenBank M13422.1) (57) using Geneious (Biomatters). Mutations were classified as transitions, transversions, deletions, insertions, or complex (multiple changes). Duplicate mutations at the same site within an individual tissue were excluded to account for clonal expansion of sibling mutations. More information on primers and methods is provided in SI Materials and Methods.

**gpt Assay and Sequencing Analysis.** The 6-TG selection assay was performed as previously described (24, 56). Briefly, phages rescued from murine genomic DNA were transfected into *E. coli* YG6620 expressing Cre recombinase. Infected cells were cultured on plates containing chloramphenicol (Cm) and 6-TG for 3 d until 6-TG-resistant colonies appeared. To confirm the 6-TG-resistant phenotype, colonies were restreaked on plates containing Cm and 6-TG. Confirmed 6-TG-resistant colonies were cultured, and a 739-bp DNA product containing the gpt gene was amplified by PCR. DNA sequencing of the gpt gene was performed by the Biopolymers Facility at Harvard Medical School (Boston, MA) with Amplicon markers (Agencourt) and a 3730XL DNA Analyzer (Applied Biosystems). Sequences were aligned with the *E. coli* gpt gene (GenBank M13422.1) (57) using Geneious (Biomatters). Mutations were classified as transitions, transversions, deletions, insertions, or complex (multiple changes). Duplicate mutations at the same site within an individual tissue were excluded to account for clonal expansion of sibling mutations. More information on primers and methods is provided in SI Materials and Methods.

**Statistical Analysis.** Two-way ANOVA followed by Bonferroni posttests were used to analyze GHA1 and mRNA expression values. Student two-tailed t tests were used to analyze mutant and mutation frequency data and serum IgG isotypes. Poisson distribution analysis was used to determine hotspots at a 99% confidence level (58). For some analyses, age-matched controls of both sexes were grouped when no statistical differences were detected between sexes. Analyses were done with GraphPad Prism, version 4.0, or Microsoft Excel 2002. P < 0.05 was considered significant.

**Acknowledgments.** We thank Suresh Kumar Muthupalani for help with the histological images and Laura J. Trudel for assistance with the manuscript and figures. This study is dedicated in loving memory of David Schauer, a mentor and a friend, for his contribution in the design and analysis of this work. This work was supported by National Institutes of Health Grants R01AI037750 and P01-CA026731 and Massachusetts Institute of Technology Center for Environmental Health Sciences Program Project Grant P30-ES02109.


*Sheh et al.*