Short-term infection with \textit{Helicobacter pylori} and 1 week exposure to metronidazole does not enhance gastric mutation frequency in transgenic mice

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The aim of this study was to determine whether exposure of \textit{Helicobacter pylori}-infected mice to metronidazole resulted in the delivery of mutagenic compounds to the gastric epithelium via the oxygen-insensitive NADPH nitroreductase (RdxA) of \textit{H. pylori}. C57BL/6 transgenic mice containing the lambda/lacI transgene were inoculated with peptone trypsin broth, \textit{H. pylori} SS1 or SS1-\textit{rdxA}\(^-\), an SS1-derived mutant in \textit{rdxA}. Twelve weeks after inoculation, the mice were treated for 7 days with a control solution or with the mouse equivalent of a human dose of metronidazole 1 g od. Three weeks after completion of treatment, the animals were killed and mutations in the target \textit{lacI} gene assessed by a transgenic mutagenesis assay system. There was no increase in \textit{lacI} mutations in cells harvested from mice infected with \textit{H. pylori} and/or exposed to metronidazole. These data suggest that short-term infection with \textit{H. pylori} and exposure to metronidazole does not enhance the mutation frequency in the gastric cells of mice. Whether chronic infection and/or repeated exposure to metronidazole or other nitroaromatic compounds causes genetic damage to gastric epithelial cells remains to be determined.

Introduction

\textit{Helicobacter pylori} is a Gram-negative, microaerobic, spiral bacterium that colonizes the human stomach.\(^1\) There is now considerable evidence that infection with \textit{H. pylori} is an important aetiological factor in the development of gastric carcinoma, and this organism has been classified as a group I carcinogen by the World Health Organization/International Agency for Research on Cancer.\(^2\)–\(^5\) Although several mechanisms—including the inflammatory response, cell proliferation, altered host metabolism and exposure to exogenous carcinogens—are likely to interact in the neoplastic progression to gastric carcinoma remain unknown.

The 5-nitroimidazole, metronidazole, is used to treat protozoal and anaerobic bacterial infections.\(^6\) The antimicrobial action of this group of drugs is dependent on the reduction of the nitro group to the nitro radical anion, and nitroso and hydroxylamine derivatives, and these have been shown to cause DNA degradation and strand breakage.\(^6\) The activity of nitroimidazoles against microaerobic bacteria, such as \textit{H. pylori}, is also dependent on reduction of the parent compound.\(^7\)–\(^9\) Recently it has been demonstrated that susceptibility to metronidazole in \textit{H. pylori} is, in part, due to the activity of an oxygen-insensitive NADPH nitroreductase (encoded by the gene \textit{rdxA}, designated HP0954 in the \textit{H. pylori} genome database),\(^10\) which reduces metronidazole to its active form.\(^11\) The RdxA protein is a homologue of the classical nitroreductase of enteric bacteria that is capable of reducing nitroaromatic compounds, including metronidazole.\(^12\) Using the Ames test, it has been demonstrated that this enzyme is capable of reducing metronidazole to mutagenic end-products in \textit{Salmonella typhimurium}.\(^13\) Moreover, mutational inactivation of its nitroreductase rendered this bacterium resistant to the mutagenic effects of nitro-containing compounds.\(^13\) It has therefore been proposed that repeated exposure of \textit{H. pylori}-infected individuals to metronidazole and other
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nitroaromatic compounds might result in the delivery of mutagenic compounds to the gastric epithelium via the oxygen-insensitive NADPH nitroreductase of *H. pylori*.11

Using transgenic rodent mutagenesis assays it is now possible to assess the genetic toxicity of test compounds in vivo and these systems allow the detection of spontaneous and mutagen-induced mutations in a variety of tissues.14–16 The Big Blue transgenic mouse assay system employs a recoverable transgenic lambda phage shuttle vector containing bacterial target (lacI) and reporter (the α-portion of lacZ) genes. Mutations in the lacI gene (which encodes the Lac repressor protein) allow transcription of α-lacZ, resulting in production of β-galactosidase, which is able to cleave 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to generate a blue colour. After treating the animal with the test compound, the shuttle vector is rescued from murine genomic DNA and packaged into viable lambda phage particles. These are used to infect an *Escherichia coli* strain, so allowing screening of the lacI mutations that were generated in the mice. The ratio of blue phage plaques to the total number of plaques is a measure of the mutation frequency and hence the genetic toxicity of the test compound in the tissue of interest.

The aim of this study was to determine whether exposure of *H. pylori*-infected mice to metronidazole resulted in genetic damage to gastric tissue via the oxygen-insensitive NADPH nitroreductase RdxA. C57BL/6 mice containing the lambda/lacI transgene were used to assess the frequency of mutational events induced in the stomach after short-term infection with the *H. pylori* SS1 strain and exposure to metronidazole.

**Materials and methods**

**Bacterial strains and growth conditions**

*H. pylori* SS1 is a mouse-adapted strain originally isolated from a patient with peptic ulcer disease.17 *H. pylori* SS1-rdxA<sup>A</sup> is a mouse-derived *H. pylori* SS1 isolate that is resistant to metronidazole (MIC 32 mg/L); it contains frameshift mutations at positions 90 and 159 of the rdxA gene, resulting in the creation of two translational stop codons within the gene.18 *H. pylori* strains were routinely cultured on blood agar (Blood Agar Base no. 2; Oxoid, Lyon, France) supplemented with 10% horse blood (bioMérieux, Marcy l’Étoile, France) and the following antibiotics: vancomycin (Dakota Pharmaceuticals, Creteil, France) 10 mg/L, polymyxin (Pfizer Laboratories, Orsay, France) 2.5 IU/L, trimethoprim (Sigma Chemicals, Saint-Quentin Fallavier, France) 5 mg/L and amphotericin B (Bristol-Myers Squibb, Paris, France) 4 mg/L. The plates were incubated at 37°C under microaerobic conditions in an anaerobic jar (Oxoid) with a carbon dioxide generator (CampyGen, Oxoid) without catalyst.

*E. coli* strain SCS-8 [recA1, endA1, mrcA, Δ(argF-lac), U169, ϕ80dlacZΔM15 Tn10(tet<sup>R</sup>)] was obtained from Stratagene (La Jolla, CA, USA) and used for screening lacI mutations.

**Infection of mice with *H. pylori* SS1**

Six-week-old specific-pathogen-free C57BL/6 mice carrying the lambda/lacI transgene (Big Blue transgenic mice; Stratagene) were housed in polycarbonate cages in isolators and fed a commercial pellet diet with water ad libitum. All animal experimentation was performed in accordance with institutional guidelines. Mice were inoculated intragastrically with a suspension of *H. pylori* SS1 (n = 9; Table) or SS1-rdxA<sup>A</sup> (n = 8), which had been harvested directly from 48 h plate cultures into peptone tryptic broth (Organotéchnique, La Courneuve, France). Each animal was inoculated with 100 µL of a suspension of 10<sup>6</sup> cfu/mL (equivalent to 100 times the 100% infectious dose) on two consecutive days. This was administered with polyethylene catheters (Biotrol, Paris, France) attached to 1 mL disposable syringes. Control groups of mice (n = 10) were given peptone tryptic broth alone.

**Antimicrobial chemotherapy**

Metronidazole was administered to mice 12 weeks after infection (Table). All solutions were administered intragastrically in a final volume of 100 mL via polyethylene catheters as described previously. The mice in groups 1 (n = 5), 2 (n = 5) and 3 (n = 4) were treated for 7 days with a control solution of 10% methanol. The mice in groups 4 (n = 5), 5 (n = 4) and 6 (n = 4) were treated once daily for 7 days with 0.513 mg metronidazole (Rhône-Poulenc Rorer, Vitry sur Seine, France) suspended in 10% methanol. This dose is the equivalent, by body weight, of 1 g metronidazole in humans. In a parallel experiment, the effects of a reference mutagen were assessed and compared with those of metronidazole.20 The compound tested was a nitrofuran derivative (R7000), which is structurally related to metronidazole.20 Mice were treated with R7000 (1.5 mg; n = 4), metronidazole (1.0 mg; n = 3) or control solution (n = 3) once daily for 5 days.

**Assessment of *H. pylori* infection in mice**

The animals were killed 3 weeks after completion of treatment, corresponding to the optimal time for expression of mutations.21 The stomach of each mouse was removed and serum was recovered in Sarstedt microtubes (Sarstedt, Nümbrecht, Germany). Serum samples were tested for *H. pylori* antigen-specific IgG antibody by a previously described enzyme-linked immunoabsorbent assay technique.22 Briefly, 96-well Maxisorb plates (Nunc, Kamstrup, Denmark) were coated with 25 µg of a sonicated whole-cell extract of *H. pylori* SS1. Serum
samples were diluted 1/100 and were added in 100 μL aliquots to coated microtitre wells. To allow for non-specific antibody binding, samples were also added to uncoated wells. Bound *H. pylori*-specific antibodies were detected by using biotinylated goat anti-mouse immunoglobulin and streptavidin–peroxidase conjugate (Amersham, Les Ulis, France). The readings for uncoated wells were subtracted from those of the respective test samples. A cut-off value was determined from the mean absorbance value ± 2 s.d. for the corresponding samples from naive uninfected mice. Samples with absorbance readings greater than this cut-off value were considered positive for *H. pylori*-specific antibodies.

**Isolation of DNA**

Genomic DNA was isolated from the stomachs of mice using the Big Blue DNA isolation kit (Stratagene) according to the manufacturer’s instructions. Tissue (c. 100 mg) was quickly disaggregated and digested with proteinase K. The DNA was extracted using phenol–chloroform and precipitated in 100% ethanol before being resuspended in a suitable volume (200–400 μL) of Tris–EDTA buffer (10 mM Tris–HCl pH 7.6, 1 mM EDTA). The genomic DNA was allowed to dissolve at room temperature for 24 h before storage at 4°C.

**Packaging and plating of phage**

The transgenic lambda phage shuttle vector containing the *lacI* gene was recovered from individual genomic DNA samples using the Transpack packaging extract (Stratagene) according to the manufacturer’s instructions. DNA extracts from individual mice were analysed separately. Packaged phage particles were preadsorbed to SCS-8 *E. coli* cells (Stratagene) for 15 min at 37°C, mixed with molten (48°C) NZY top agarose (7%) containing 1.5 mg/mL X-gal and poured into square plates (25 cm × 25 cm) containing 250 mL NZY agar. The plaque density was limited to a maximum of 15 000 pfu/plate. Following incubation for 18 h at 37°C, the plates were examined for the presence of blue mutant plaques on a background of non-mutant colourless plaques using a light box with a red transparency to enhance colour contrast. After determination of the number of blue mutant plaques, the total number of plaques was determined by counting three representative squares (each representing 1/100 of the total plate area). Comparison with positive control mutant plaques was carried out for each experiment. The mutant frequency was calculated by dividing the number of mutant blue plaques by the total number of plaques plated.

**Statistical analysis**

Differences in mutation frequency between the different groups of mice were determined using a two-tailed *t*-test. A *P* value ≤0.05 was considered significant.

**Results**

In groups 1 and 4, none of the 10 mice inoculated with peptone trypsin broth had serological evidence of infection with *H. pylori* at the time of death (Table). In contrast, all nine SS1-inoculated mice in groups 2 and 5, and all eight SS1-*rdxA*–inoculated mice in groups 3 and 6, tested positive for *H. pylori* antigen-specific IgG.

The mean number of plaques screened per animal was 184 000. The average mutation frequency for control animals (group 1) was 10.65 × 10⁻⁵, with a range of between 7.63 × 10⁻⁵ and 13.30 × 10⁻⁵ in individual animals (Table). There was no significant difference between the mutation frequency for the control animals and those infected with *H. pylori* SS1 or SS1-*rdxA* (groups 2 and 3, respectively). Mutation frequencies were also similar for control animals.

**Table.** Mutations in the *lacI* gene induced in gastric cells from C57BL/6 transgenic mice after exposure to *H. pylori* SS1 and metronidazole

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Inoculating suspension</td>
<td>PTB⁴</td>
<td>SS1</td>
<td>SS1-<em>rdxA</em>-</td>
<td>PTB</td>
<td>SS1</td>
<td>SS1-<em>rdxA</em>-</td>
</tr>
<tr>
<td>Treatment</td>
<td>control⁵</td>
<td>control</td>
<td>control</td>
<td>MTZ⁶</td>
<td>MTZ</td>
<td>MTZ</td>
</tr>
<tr>
<td>Infection with <em>H. pylori</em></td>
<td>0/5</td>
<td>5/5</td>
<td>4/4</td>
<td>0/5</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Total pfu (&lt;10⁵)</td>
<td>10.33</td>
<td>9.60</td>
<td>6.75</td>
<td>9.72</td>
<td>5.40</td>
<td>7.79</td>
</tr>
<tr>
<td>Mutant plaques</td>
<td>110</td>
<td>69</td>
<td>56</td>
<td>103</td>
<td>46</td>
<td>48</td>
</tr>
<tr>
<td>Mutant frequency (&lt;10⁻⁵)</td>
<td>10.65 ± 1.04</td>
<td>7.19 ± 1.18</td>
<td>8.29 ± 1.32</td>
<td>10.59 ± 2.08</td>
<td>8.52 ± 1.5</td>
<td>6.16 ± 0.94</td>
</tr>
</tbody>
</table>

⁴PTB, peptone trypsin broth.
⁵Control, 10% methanol.
⁶MTZ, metronidazole in 10% methanol.
⁷Proportion of mice infected with *H. pylori* at death.
and uninfected mice exposed to metronidazole (group 4). The average mutation frequency for mice infected with *H. pylori* SS1 and treated with metronidazole (group 5) was $8.52 \times 10^{-5}$, with a range of between $5.13 \times 10^{-5}$ and $11.20 \times 10^{-5}$ in individual animals (Table). There was no significant difference between the mutation frequency for these mice and for either control animals (group 1) or for mice infected with SS1-nudA$^+$ and exposed to metronidazole (group 6).

In experiments comparing the effects of metronidazole with a reference mutagen, R7000, the average mutation frequencies were as follows: control solution, $7.18 \times 10^{-5}$; metronidazole, $10.59 \times 10^{-5}$; R7000, $33.00 \times 10^{-5}$. The mutation frequency for metronidazole in this experiment was similar to that observed in uninfected mice exposed to metronidazole (group 4; $10.59 \times 10^{-5}$). The mutation frequency of R7000 was approximately five times that of the control solution. A 1.5-fold increase in the mutation frequency of a test compared with a control compound is considered a positive result using this assay.22

**Discussion**

There is considerable epidemiological evidence that *H. pylori* infection is associated with the development of gastric cancer2-5 and it has been postulated that *H. pylori* colonization induces superficial gastritis that may lead to chronic atrophic gastritis, intestinal metaplasia, dysplasia and ultimately adenocarcinoma of the stomach.23 A recent meta-analysis of 19 cohort and case–control studies found a summary odds ratio of 1.92, suggesting that the risk of stomach cancer is approximately doubled among infected individuals.24 In addition, some, though not all, epidemiological studies have shown that patients with preneoplastic and neoplastic gastric epithelial lesions are more likely to be infected by CagA-positive strains, which may be related to the enhanced inflammatory potential of these strains.25-27 However, the link between *H. pylori* and gastric carcinoma remains unclear and the development of the intestinal type of gastric cancer is likely to be a gradual process dependent on the interplay between a number of factors including a high salt diet, ascorbic acid concentrations and N-nitroso compounds produced by anaerobic bacteria from dietary sources.28-32 Host genetic factors are also likely to be important in determining the immune response to *H. pylori* infection and the propensity to develop gastric atrophy and hypochlorhydria in response to infection.33,34 It has recently been demonstrated that *H. pylori* infection enhances the carcinogenic action of primary carcinogens such as N-methyl-N-nitrosourea and N-methyl-N'-nitro-N-nitrosoguanidine in Mongolian gerbils.35,36 and it has been proposed that *H. pylori* may reduce metronidazole to highly active derivatives capable of inducing mutations in the host’s gastric epithelium.11

The Big Blue transgenic mouse model has been extensively validated for use in mutagenesis assays. It has been used to confirm the mutagenic potential of microorganisms (the liver fluke) and numerous group 1 and group 2 chemical carcinogens, including benzene, cyclophosphamide and N-methyl-N-nitrosourea.22 The sensitivity of this assay is such that a 1.5-fold increase in the mutation frequency of a test compared with a control compound is considered a positive result.22 This assay system was validated in our laboratory using the 5-nitrofuran, R7000.20 The mutation frequency of R7000 was approximately five-fold higher than the control solution. Using the Big Blue transgenic mouse assay system we have demonstrated that short-term infection with *H. pylori* and exposure to metronidazole does not appear to increase the mutation frequency in the murine stomach. Our results were obtained using a relatively short period of colonization (15 weeks) and only one course of metronidazole (the mouse equivalent of 7 g) was administered. Although relatively small increases in mutation frequency might have been detected by screening more plaque-forming units in a larger number of animals,37 our results failed to show any trend towards an increase in mutation frequency in *H. pylori*-infected mice.

The lack of enhancement of mutation frequency in mice may in part be explained by inter-species variation in the response to *H. pylori* infection and in particular the role of chronic inflammation in the development and progression of mucosal damage. Mice inoculated with *H. pylori* SS1 develop life-long infection, with gastric bacterial loads similar to those found in humans, and this model has been standardized for antimicrobial studies, vaccine development and studies in pathogenesis.17 We have previously used the *H. pylori* SS1 mouse model of infection to study the evolution of metronidazole resistance by *H. pylori* and have demonstrated that this is a suitable system for studying interactions between the bacterium and antimicrobial agents in vivo.18,38,39 However, certain limitations of the mouse model of infection may explain why no increase in mutation frequency was observed in *H. pylori*-infected animals. One problem of using a mouse model is that the induced pathological changes are generally less severe than in other animal models.40 In particular, while the development of adenocarcinomas has been reported in the stomachs of Mongolian gerbils infected with *H. pylori*,41 such pathological changes have not been observed in *H. pylori*-infected native mice. In addition, it has become clear that the degree of inflammation induced by *H. pylori* is dependent on both the bacterial strain and the host.42 In humans, chronic *H. pylori* infection may damage gastric barrier function, stimulate gastric cell proliferation and induce cellular DNA damage.43-45 However, recent work in mice has suggested that *H. pylori* SS1 may induce relatively mild inflammatory changes in the murine gastric epithelium.42 *H. pylori* SS1 (which has the cagA$^+$ and vacA s2-m2 genotype) induced neither vacuole formation in HeLa cells nor interleukin-8 production in KATO III cells, and failed to induce chronic inflammation in C57BL/6 mice (although
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such changes were described in the initial characterization of this model.\(^1,42\) Taken together, these observations suggest that the pathological changes induced in C57BL/6 mice may be relatively mild and unlikely to result in mutational changes in the murine stomach.

Acquired genome instability, which is typical of the phenotypic expression of precancerous lesions, generally precedes neoplastic clonal expansion, but whether chronic *H. pylori* infection is responsible for genomic instability in a subset of cases of *H. pylori*-infected individuals remains unclear. Although one study has demonstrated that *H. pylori* can induce p53 tumour suppressor gene mutations,\(^46\) a detailed analysis of early gastric carcinomas for mutations in certain oncogenes found no correlation between the molecular alterations and *H. pylori* status.\(^47\) The absence of a significant difference in the molecular profile between *H. pylori*-positive and *H. pylori*-negative early carcinomas suggests the possibility that the acquisition of molecular alterations in early gastric cancer may occur via an *H. pylori*-independent pathway. Our results could be interpreted as supporting the hypothesis that *H. pylori* is a factor facilitating the multifactorially determined process of gastric carcinogenesis, but by itself does not initiate the oncogenic process.\(^47\) However, because the outcome of *H. pylori* infection results from a complex interaction between the inherent genetic susceptibility of the host and bacterial factors modified by environmental exposure to various other carcinogens, further work is needed before the exact role of *H. pylori* in gastric carcinogenesis can be defined. Further experiments are under way in our laboratory to determine whether the gastric mutation frequency is affected by long-term colonization with *H. pylori*, repeated doses of metronidazole and exposure to other potentially oncogenic cofactors.

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