Gastric Lesions and Immune Responses caused by Long-term Infection with Helicobacter heilmannii in C57BL/6 Mice

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Summary

Helicobacter heilmannii is a gastric micro-organism that can induce gastritis and B-cell MALT (mucosa-associated lymphoid tissue) lymphoma in mice, in a host-dependent manner. The present study was designed to examine gastric lesions and immune responses caused by intragastric H. heilmannii infection of an inbred mouse strain, C57BL/6. Long-term infection led to the formation of gastric nodules and increased mucosal thickness of the stomach, due to gastric epithelial proliferation. Infection also induced the formation of lymphoid follicles in the corpus mucosa and submucosa. The follicular cells were mainly CD45R+ cells that did not produce immunoglobulin. However, scattered in the lamina propria and corpus submucosa, numerous IgA+ cells were found in infected mice, but not in control mice. RT-PCR results showed that H. heilmannii infection led to increased mRNA expression for IFN-γ (a Th1 cytokine) and IL-10 (a Th2 cytokine) in the mouse stomach, suggesting that both Th1 and Th2 responses are associated with H. heilmannii infection. The mRNA of other cytokines and chemokines (IL-1β, IL-12p40, TNF-α, MCP-1, KC and MIP-2) was also increased by infection.

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Introduction

Most human helicobacter gastric disorders are caused by Helicobacter pylori. However, Helicobacter heilmannii can also infect and colonize human gastric mucosa at a low rate (0.5–6%) (O’Rourke et al., 2002) and lead to gastritis (Mazzucchelli et al., 1993), gastroduodenal ulcer (Debongnie et al., 1998), or malignant lymphoma (Stolte et al., 1997). Since infection by H. heilmannii has also been reported in the gastric mucosa of various mammals including cats, dogs, pigs and nonhuman primates (De Groote et al., 1999; O’Rourke et al., 2004; Priestnall et al., 2004), it is strongly suspected to be a zoonotic agent.

Studies on H. heilmannii have been impeded by inability to culture the organism on artificial media. Nonetheless, several pathological and immunological studies have been made in different mouse strains, with inocula consisting of homogenized stomach samples or gastric parietal cell cultures from H. heilmannii-infected mice (Peterson et al., 2001; Cinque et al., 2002; Nakamura et al., 2002; Park et al., 2003; O’Rourke et al., 2004).

Gastric lesions and immune responses in Helicobacter infections are influenced by bacterial strain and host strain (Mohammadi et al., 1996; Sakagami et al., 1996, 1997; Court et al., 2003; Thompson et al., 2004). O’Rourke et al. (2004) showed that long-term H. heilmannii infection of BALB/c mice caused B-cell MALT lymphoma in which bacterial isolates originating from different hosts produced forms of
disease that differed in severity. Moreover, in a study by Sakagami et al. (1996), Helicobacter felis and H. pylori infection caused moderate to severe chronic active gastritis in C57BL/6 and C3H/He mouse strains, but only mild gastritis in BALB/c and CBA mice. The difference in response between C57BL/6 and BALB/c may be due to a difference in the dominant T helper cell type in these strains, C57BL/6 mice being Th1 phenotype dominant and BALB/c mice Th2 dominant (Roth et al., 1999; Ferrero et al., 2000).

In an earlier study (Park et al., 2003), ICR mice infected with H. heilmannii originating from a pig stomach developed inflammatory infiltration and gastric dysplasia; however, further characterization of the host response proved impossible because of the outbred genetic background of the mouse strain. Long-term H. heilmannii infection has been studied in BALB/c mice (Peterson et al., 2001; O’Rourke et al., 2004) and recently in C57BL/6 mice (Nakamura et al., 2007). The present study was designed to clarify the host response in mice of the inbred strain C57BL/6.

Materials and Methods

Preparation of Gastric Homogenates

The strain of H. heilmannii used, which originated from a pig stomach, has been maintained in the infected stomachs of ICR mice (Park et al., 2003). A partial 16S rRNA sequence of the micro-organism was submitted to the Genbank database and assigned the accession number AY517854. Stomachs were collected from 10 infected mice and washed in sterile phosphate-buffered saline (PBS; pH 7.4) to remove gastric contents. They were then cut into small pieces and homogenized gently in PBS with an electric homogenizer (Ultra-turrax T25, Ika, Staufen, Germany) for use as the inoculum.

Animals

Specific pathogen-free female C57BL/6 mice aged 5 weeks were obtained from Orient (Seongnam, Gyounggi-do, Korea). They were housed in polycarbonate cages in isolators at 22 ± 2°C and 50% humidity, fed a commercial pelleted diet with water ad libitum, and acclimatized for 1 week. Food and water were removed from the cages 6 h before inoculation. Mice (n = 67) were infected intragastrically with 0.5 ml of the gastric homogenate as described previously (Park et al., 2004b). Control mice (n = 60) were given 0.5 ml of PBS by the same route. The mice were killed at 12, 15 and 18 months post-inoculation after having been weighed. All animal experiments were performed in accordance with the Guidelines and Regulations for the Care and Use of Laboratory Animals in Seoul National University.

Sample Preparation

After euthanasia, the stomachs were removed and opened along the greater curvature and the gastric contents were gently washed out with PBS. Gross examination of the stomachs was performed and the stomachs were weighed before being fixed in 10% buffered formalin, processed in an alcohol–xylene series, and embedded in paraffin wax. Sections (2 µm) were cut and stained with haematoxylin and eosin (HE) for histological examination. Selected serial sections were stained with modified Steiner’s silver stain (Sigma, St Louis, MO, USA) to assess bacterial colonization. A sample of the stomach of each mouse killed at 18 months post-inoculation was frozen at −70°C for RNA extraction.

Histopathology

Gastric lesions (inflammation, cystic dilatation and metaplasia) were assessed in HE-stained sections. Measurements (n = 10 per tissue) of gastric mucosal thickness were obtained with a micrometer, from the luminal epithelial surface to the lamina submucosalis.

Immunohistochemistry (IHC)

Gastric tissue sections were incubated with H2O2 3% in methanol for 10 min to block endogenous peroxidase activity, and subsequently blocked with bovine serum albumin 1% in PBS for 1 h. To detect gastric B and T lymphocytes, the sections were treated overnight at 4°C with rat anti-mouse CD45R (Serotec, Oxford, UK) diluted 1 in 200. Mouse thymus was used as a positive control for CD3 antibody. The sections were washed with Tween 20 0.05% in PBS (T-PBS) three times for 5 min and treated with horse-radish peroxidase (HRP)-conjugated mouse anti-rat IgG (Zymed, South San Francisco, CA, USA) at room temperature for 1 h. To detect proliferating cells, mouse anti-proliferating cell nuclear antigen (PCNA) (Serotec) diluted 1 in 100 was used as a primary antibody with the mouse on mouse (M.O.M.) Immunodetection kit (Vector Laboratories, Burlingame, CA, USA). To detect immunoglobulin-secreting B cells, the sections were treated overnight at 4°C with HRP-conjugated anti-mouse IgA, IgG1, IgG2b and IgM antibodies (Zymed) diluted 1 in 200. Peroxidase activity was evaluated with 3, 3′-diaminobenzidine (Vector) as the chromogen. Finally, the sections
were counterstained with haematoxylin for 5 s, washed in tap water, and mounted.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA extraction from gastric samples and cDNA synthesis were performed as described previously (Park et al., 2004a). The PCR was performed with the AccuPower® PCR PreMix kit (Bioneer, Daejeon, Korea) and specific primer sets for cytokines and chemokines. The primer sequences for IFN-γ, IL-1β, IL-5, IL-12p40, TNF-α, KC, MCP-1, MIP-2 and β-actin have been published (Park et al., 2004a). The primer sets for IL-6 and IL-10 were as follows: IL-6, F: 5'-CATGTTCTCTGGGAAATCGTGG-3', R: 5'-AAC TGATATGCTTAGCATAACGCAC-3', and IL-10, F: 5'-AGCTGGACAACATACTGCTAACCGA-3', R: 5'-TTTCCAAAGGAGTTGTTCCGTA G-3'. PCR conditions were as follows: 3 min of denaturation at 95°C followed by 35 cycles consisting of 30 s denaturation at 95°C, 30 s annealing at 53°C, and 1 min extension at 72°C before a final 7-min extension step at 72°C. The PCR product was detected by electrophoresis of 9 μl of reaction solution in 1.5% agarose gel, followed by staining with ethidium bromide. The bands of the PCR product were “visualized” on a UV transilluminator and their intensities were analysed by 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA). Arbitrary units were determined by dividing the density of each gene by the density of β-actin.

Statistics

Significant differences between the experimental and control groups were determined by Student’s t-test.

Results

Gross Lesions and Relative Stomach Weight (RSW)

H. heilmannii infection was confirmed in all inoculated mice by modified Steiner’s silver staining, long, helical bacteria being demonstrated in the lumen of gastric glands of infected mice, but not in control mice (data not shown).

At necropsy, gross pathological changes in the infected mice, but not in any of the control mice,

![Image](image-url)

Fig. 1A–C. Macroscopical view of the stomachs of an age-matched control (A) and a H. heilmannii-infected mouse (B) killed 12 months after inoculation (arrows indicate gastric nodules). The relative stomach weight (C) was obtained by dividing stomach weight by body weight. *P < 0.05, **P < 0.01, ***P < 0.001.
consisted of white to greyish nodules and an increased thickness of the mucosa of the gastric body at all experimental timepoints (Fig. 1A and B). No ulcerative or haemorrhagic lesions were detected in infected mice. The RSW, which was measured by dividing the stomach weight by the body weight, was significantly higher in infected mice than in control mice at all experimental timepoints. This difference increased gradually with time (Fig. 1C).

Histological Findings

The gastric nodules in the corpus mucosa of the *H. heilmannii*-infected mice (Fig. 1B) represented foci of gastric hyperplasia (Fig. 2B). The severity of epithelial hyperplastic lesions increased with the duration of infection. In addition, microscopical measurement showed that, at all timepoints, the corpus mucosa was significantly thicker in infected mice than in the controls (Table 1). From 15 months after infection, in the corpus of some infected mice the glandular epithelium intruded into the submucosa and formed glandular structures accompanied by lymphocytic infiltration (Fig. 2C). Moreover, lympho-epithelial lesions were observed in the corpus mucosa of some infected mice (Fig. 2D). However, none of these lesions were found in the stomachs of control mice (Fig. 2A). Other findings were cystic changes and mucous cell metaplasia, which were found more frequently in infected mice than in control mice (Table 1). Moreover, at all timepoints the number of PCNA-positive cells in the corpus mucosa was significantly higher in infected mice than in the controls (Fig. 3).

A further feature in the infected mice, but not in the controls, was the presence of lymphoid follicles in the corpus mucosa and submucosa. Most lymphoid follicles were composed of small, dark lymphocytes (Fig. 4A), but a few were composed of large lymphoblasts and neutrophils (Fig. 4B and C). A mild neutrophilic infiltration was observed in the lamina propria and corpus submucosa of some mice, but there was no significant difference in this response between the control and infected groups (data not shown). Lymphocytes scattered in the lamina propria

![Fig. 2A-D](Fig. 2A–D. Histopathological findings. The normal structure of the corpus mucosa from an uninfected, age-matched, control mouse (A). Marked glandular hyperplasia with mucous cell metaplasia in a *H. heilmannii*-infected mouse killed 12 months after infection (B). Newly formed glandular structures accompanied by lymphocytic infiltration in the corpus submucosa of an infected mouse, 15 months after infection (C). A lympho-epithelial lesion in the corpus mucosa of an infected mouse, 15 months after infection (D). HE. Bars, 160 μm.)
or submucosa were strongly immunolabelled for CD45R+, and lymphoid follicular cells were weakly labelled. However, the proportion of CD45R+ cells in lymphoid follicles varied markedly between follicles (Table 2). No CD3+ cells were found.

Table 1
Thickness of mucosa and summary of histopathological findings

<table>
<thead>
<tr>
<th>Observation</th>
<th>12 months</th>
<th>15 months</th>
<th>18 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Infected</td>
<td>Control</td>
</tr>
<tr>
<td>Thickness of mucosa*</td>
<td>0.433 ± 0.068</td>
<td>0.572 ± 0.111†</td>
<td>0.450 ± 0.068</td>
</tr>
<tr>
<td>Histopathology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystic change</td>
<td>5/16</td>
<td>12/19</td>
<td>5/20</td>
</tr>
<tr>
<td>Moderate</td>
<td>(5/5)</td>
<td>(11/12)</td>
<td>(4/5)</td>
</tr>
<tr>
<td>Severe</td>
<td>(0/5)</td>
<td>(1/12)</td>
<td>(1/5)</td>
</tr>
<tr>
<td>Mucous cell metaplasia</td>
<td>2/16</td>
<td>13/19</td>
<td>4/20</td>
</tr>
<tr>
<td>Moderate</td>
<td>(1/2)</td>
<td>(6/13)</td>
<td>(3/4)</td>
</tr>
<tr>
<td>Severe</td>
<td>(1/2)</td>
<td>(7/13)</td>
<td>(1/4)</td>
</tr>
</tbody>
</table>

†P < 0.001.
Numerator = numbers of mice affected; denominator = numbers of mice examined.
*Measured with a micrometer from tip of epithelial surface to the lamina subglandularis (mm).

Immunohistochemical Detection of Immunoglobulin-secreting Cells

IgA+ cells were mainly scattered in the lamina propria, especially of the upper gastric mucosa, and the submucosa in infected mice (Fig. 5A and B). The

Fig. 3A–C. Immunohistochemical detection of PCNA-positive cells 12 months after infection in the corpus mucosa of control (A) and infected mice (B) (A, B; ×200). PCNA-positive cells were counted in 10 randomly selected columns from one mouse and the result was expressed as the mean ± SD per column (C). ***P < 0.001.
number of IgA+ cells was significantly higher in infected mice than in control mice (Fig. 5C). However, IgG1-, IgG2b- and IgM-positive cells were rarely found in the gastric mucosa of control or infected mice (data not shown). No immunoglobulin-secreting cells were found in lymphoid follicles.

RT-PCR

The mRNA expression of several inflammatory mediators was examined by RT-PCR in mice killed 18 months after inoculation. The mRNA levels of the cytokines IFN-γ, IL-1β, IL-10, IL-12p40 and TNF-α were elevated in infected mice, but those of IL-5 and IL-6 were not (Fig. 6). The mRNA levels of the chemokines KC, MIP-2 and MCP-1 were also greatly increased in infected mice as compared with the levels in the control mice (Fig. 6).

Discussion

In this study, C57BL/6 mice were infected intragastrically with H. heilmannii (originating from a pig stomach) and examined at 12, 15 and 18 months after inoculation. Macroscopical examination revealed

Table 2

The proportion of CD45R+ cells in lymphoid follicles in H. heilmannii-infected mice

<table>
<thead>
<tr>
<th>Infection period (months)</th>
<th>CD45R+ (%)</th>
<th>Mucosa</th>
<th></th>
<th>Submucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;25%</td>
<td>25–50%</td>
<td>50–75%</td>
<td>&gt;75%</td>
</tr>
<tr>
<td>12</td>
<td>13.5</td>
<td>10.8</td>
<td>41.9</td>
<td>33.8</td>
</tr>
<tr>
<td>15</td>
<td>6.6</td>
<td>16.4</td>
<td>39.3</td>
<td>37.7</td>
</tr>
<tr>
<td>18</td>
<td>7.8</td>
<td>15.5</td>
<td>31.1</td>
<td>45.6</td>
</tr>
</tbody>
</table>

The proportion of CD45R+ cells in lymphoid follicles was examined as described by Ferrero et al. (2000).
gastric nodules and increased mucosal thickness in the gastric body of the infected mice at all experimental timepoints. Histological examination showed that these nodules were composed of hyperplastic epithelial cells, a novel finding. In a study by O’Rourke et al. (2004) in which BALB/c mice were infected with *H. heilmannii* originating from various hosts, large nodules were produced in the region of the cardia in the gastric body mucosa along the greater curvature; these nodules were shown histopathologically to consist of large lymphoid aggregates. Nakamura et al. (2007) also demonstrated destruction of glandular

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![Fig. 5A-C. Immunohistochemical identification of IgA+ cells in the corpus mucosa. A large number of IgA+ cells were found in the upper corpus mucosa of infected mice (B), but not in control mice (A) (A, B; ×100). Fig. 5C shows the scores for IgA+ cells counted in five randomly selected areas (×400) and expressed as follows: 0 = none, 1 = 1–10, 2 = 11–20, 3 = 21–50, 4 = 51–100, 5 = over 100. Data were expressed as mean ± SD. ***P < 0.001.](image)

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![Fig. 6. RT-PCR analysis of mRNA expression of inflammatory mediators. Arbitrary units were obtained by dividing the density of each gene PCR product by the density of the β-actin PCR product. *P < 0.05, **P < 0.01, ***P < 0.001.](image)
tissue and the appearance of lympho-epithelial lesions with low-grade MALT lymphoma in the stomach of “Candidatus _Helicobacter heilmannii_”-infected C57BL/6 mice, but did not mention gastric epithelial hyperplasia. This discrepancy in nodule composition may have been due to the use of different mouse strains. Other studies suggest that BALB/c mice are particularly susceptible to B-cell lymphomagenesis in response to long-term infection with _H. felis_ (Enno et al., 1995; Ferrero et al., 2000). Moreover, the gastric lesions in BALB/c mice in response to _H. felis_ infection were characterized by the formation of gastric lymphoid follicles, whereas the nodules in other strains (C57BL/6, SJL, and C3H mice) were characterized by significant modification of gastric epithelium (Mohammadi et al., 1996; Sakagami et al., 1996, 1997). Bacterial strain differences should also be considered as a possible reason for differences in gastric lesions produced by _H. heilmannii_ infection. O’Rourke et al. (2004) demonstrated variations in the severity of B-cell MALT lymphoma produced by _H. heilmannii_ strains originating from different hosts (bobcat, mandrill monkey, macaque and man). In addition, the RSW was significantly higher in _H. heilmannii_-infected mice than in control mice, the difference increasing with time. The number of PCNA-positive gastric epithelial cells was greater in infected mice than in the controls. These results demonstrate that the increased RSW in infected mice was due to an increased thickness of the gastric mucosa as a result of epithelial cell proliferation.

Several studies showed that the formation of lymphoid follicles or B-cell MALT lymphoma was the characteristic lesion of _H. heilmannii_ infection in mice (Peterson et al., 2001; Park et al., 2003; O’Rourke et al., 2004), although its severity varied. In the present study, long-term infection of C57BL/6 mice with _H. heilmannii_ induced the formation of lymphoid follicles in the corpus mucosa and submucosa. The follicular cells consisted mainly of CD45R+ cells, which are considered to be of B-cell origin, but the proportion of these cells in the total population varied between follicles. In addition, no CD3+ cells (T cells) were found in the tissues examined. Peterson et al. (2001) showed that CD4+ T cells increased in number in the gastric mucosa of _H. heilmannii_-infected BALB/c mice. In the study by Nakamura et al. (2007), however, most of the aggregated lymphocytes were B220-positive and CD3-negative cells. Clarification is required as to whether these differences are due to host-specific responses of C57BL/6 and BALB/c mice or to different experimental methods and reagents.

To examine the gastric distribution of immunoglobulin-secreting cells, gastric tissues were labelled immunohistochemically for IgA, IgG1, IgG2b, and IgM. A large number of IgA+ cells were present in the lamina propria (especially of the upper mucosa) and the corpus submucosa in infected mice, but not in control mice. IgG1-, IgG2b- and IgM-positive cells were rarely found in the gastric mucosa of control or infected mice. These findings differ from (1) those of Peterson et al. (2001), who showed that IgG- and IgM-positive cells were increased in number in the gastric mucosa of BALB/c mice infected with _H. heilmannii_, and (2) a study with outbred Swiss mice infected with _H. felis_, in which gastric IgA and IgG+ cells were present in increased numbers, but the number of IgM+ cells was not increased (Ferrero et al., 2000). It is likely that the type of immunoglobulin-secreting cells in _Helicobacter_ infection is also host- and bacterial strain-specific. In addition, lymphoid follicles appear not to be a major site of antibody production. Thus, no immunoglobulin-secreting cells were found immunohistochemically in lymphoid follicles.

In this study, the RT-PCR was performed to examine mRNA expression of inflammatory mediators (cytokines and chemokines) at 18 months after infection. The mRNA levels of IFN-γ (a Th1 cytokine) and IL-10 (a Th2 cytokine) were higher in the gastric tissues of infected mice than in those of control mice. However, the mRNA of IL-5 and IL-6, which may also be produced by Th2 cells, did not differ between control and infected mice. This suggests that both Th1 and Th2 responses participate in the inflammation caused by _H. heilmannii_ infection in the mouse stomach. In addition, mRNA levels of KC, MIP-2 and MCP-1 were also increased in gastric tissues of _H. heilmannii_-infected mice. KC and MIP-2 are potent neutrophil-chemoattractant factors in mice. Paradoxically, neutrophilic infiltration was observed in the gastric mucosa of both control and infected mice; its severity did not differ significantly between the two groups of mice, although it was rarely found within lymphoid follicles in the gastric mucosa of infected mice (Fig. 4C). Peterson et al. (2001) showed that neutrophilic infiltration in the gastric mucosa of BALB/c mice in response to _H. heilmannii_ infection reached a peak 2 weeks after infection and then gradually decreased, until after 12 months there was no difference between inoculated and control mice, suggesting that neutrophilic gastritis is an early event in _H. heilmannii_ infection. In the present study, the earliest examination was made 12 months after infection, by which time any neutrophil infiltration would probably have disappeared. However, mRNA levels of KC and MIP-2 remained high. To clarify this, further study is needed.

In conclusion, this study demonstrated that long-term infection with _H. heilmannii_ can induce both gastric epithelial hyperplasia and the formation of
lymphoid follicles in C57BL/6 mice. This animal model may prove helpful in studying the pathogenesis of gastric disorders caused by Helicobacter infection.

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