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Two-year follow-up of Helicobacter pylori infection in C57BL/6 and Balb/cA mice

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Helicobacter pylori infection is associated with chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and MALT lymphoma. We previously found high-grade lymphoma after 13 months' H. pylori infection in C57BL/6 mice. In this study we followed H. pylori infection by three different isolates in C57BL/6 and Balb/cA mice for 23 months. Six-week-old C57BL/6 and Balb/cA mice were infected with H. pylori strains 119p (CagA+, VacA+), SS1 (CagA+, VacA+) and G50 (CagA-, VacA-). Mice were followed at 2 weeks, 10 weeks and 23 months post-inoculation (p.i.) by culture, histopathology and serology. Strain G50 was only reisolated from mice 2 weeks p.i. There was no difference in colonization between strain 119p and SS1 at 10 weeks p.i., whereas SS1 gave 100% colonization versus 119p gave 50% 23 months p.i.. Interestingly, the inflammation score was higher in mice infected with strain 119p than with SS1 10-week p.i., and there were lymphoepithelial lesions in mice infected with strain 119p and G50 but not with SS1 at 23 months post-infection. Eight mice infected with strains 119p and G50 developed gastric lymphoma (grade 5 and 4). One C57BL/6 mouse infected with strain 119p developed hepatocellular carcinoma after 23 months. Immunoblot showed specific bands of 26–33 kDa against H. pylori in infected mice, and two mice infected with strain SS1 reacted with antibodies to the 120 kDa CagA toxin.

Conclusion: A reproducible animal model for H. pylori-induced lymphoma and possibly hepatocellular carcinoma is described. Strain diversity may lead to different outcomes of H. pylori infection.

Key words: Helicobacter pylori; lymphoma; hepatocellular carcinoma; C57BL/6 and Balb/cA mice.

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Helicobacter pylori has been implicated in the pathogenesis of most clinically important gastroduodenal diseases, such as peptic ulcer, gastric carcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. H. pylori was classified as a Class I carcinogen by the International Agency for Research on Cancer (IARC) in 1994 (1) Prospective investigations showed that infected individuals had a four-fold or higher increased risk of developing gastric cancer. This has later been related to H. pylori-induced atrophy and aging. After correction for the effects of atrophy and aging, the relative risk may be in the order of 9- or 10-fold (2).

H. pylori induces direct DNA damage (3) and tissue inflammation, cell apoptosis and proliferation (4, 5). This could provide the link between chronic H. pylori infection and development of adenocarcinoma of the stomach. Moreover, H. pylori induces intestinal-type gastric adenocarcinoma in 40% of infected Mongolian gerbils about 70 weeks post-inoculation (6, 7). H. pylori enhances glandular stomach carcinogenesis in these animals, and in mice treated with a chemical carcinogen such as sodium nitrite (8, 9). A
multistep model for the development of gastric cancer has been proposed from superficial gastritis progressing to chronic atrophic gastritis, intestinal metaplasia, dysplasia and ultimately carcinoma (10). Environmental factors usually implicated in the development of gastric cancer are high salt and nitrate intake (11), promoting cancer development, while antioxidants (ascorbic acid, β-carotene, vitamin E and C) may inhibit this development. A recent 4.5-year follow-up study in Linqu of China with one of the world’s highest rates of gastric cancer showed that the presence of H. pylori was associated with an increased risk of progression to dysplasia and gastric cancer, and the risk of progression was decreased among subjects with high vitamin C intake and high vitamin C serum levels (12). While this has been supported by in vitro studies, the doses needed are far above those achieved in gastric juice of H. pylori patients, and may hence be of limited importance in vivo (13).

We earlier reported on development of high-grade lymphoma after 13 months’ H. pylori infection in C57BL/6 mice (14). In order to elucidate the mechanisms behind malignant transformation of chronic H. pylori infections, a reproducible animal model is needed. In this study, H. pylori infections by three different strains in C57BL/6 and Balb/cA mice were followed for 23 months.

**MATERIALS AND METHODS**

_Bacterial Strains_

The _H. pylori_ strain 119p (mouse passaged from strain 119/95, a cagA+, vacA s1m1 strain originally isolated from a duodenal ulcer patient, University Hospital of Lund, Sweden) (15), SS1 (a cagA+, vacA s2-m2 strain) (16) and G50 (a CagA+, VacA-) (17) were grown on GAB-Camp agar supplemented with 10% horse serum and incubated for 48 h at 37°C under microaerophilic conditions (18). The cells were harvested in PBS, centrifuged at 3000 rpm for 10 min, and resuspended in PBS at a final concentration of 10⁵ colony-forming units (cfu)/ml.

_Animals_

Six- to eight-week-old conventional C57BL/6 and Balb/cA mice were kept in microisolated cages with a 12 h light-dark schedule and fed with rat and mouse standard diet no. 2 expanded (B&K Universal Company, Stockholm, Sweden) (9) and water ad libitum. The inoculation with bacteria was done as described previously (18). Twenty-four C57BL/6 mice were inoculated with _H. pylori_ strain 119p, SS1 and G50, and 16 Balb/cA mice were inoculated with _H. pylori_ strain 119p and SS1 (8 mice of each strain). Control animals consisting of C57BL/6 (n=8) and BALB/cA (n=8) mice were inoculated with PBS only. Gastric colonization with _H. pylori_ was assessed at 2 weeks, 10 weeks and 23 months post-inoculation (p. i.). Histopathology was examined at 10-week and 23-month p. i.

_Culture_

Half stomach biopsies were rinsed in PBS, smeared directly on GAB-Camp agar, and incubated at 37°C for 5–10 days under microaerophilic conditions (15). The presence of _H. pylori_ on the culture plates was confirmed by urease, catalase and oxidase tests, Gram staining and PCR (19). No _H. pylori_ colony on the plate was recognised as culture negative.

_Preparation of antibodies against H. pylori_

New Zealand white rabbits were injected subcutaneously with 140 μg of sonicated whole cell material of _H. pylori_ strain CCUG 17874, mixed with Freund’s adjuvant in five divided doses (day 1, 6, 18 and 24). Three weeks later the animals were bled and the sera were separated. The IgG-fraction was purified by affinity chromatography on a protein G Sepharose 4 fast flow column (Amersham Pharmacia Biotech, Uppsala, Sweden). The IgG-fraction was aliquoted and stored at −20°C.

_Histopathology and immunohistochemistry_

Murine stomach, liver, spleen and kidney tissues were fixed in 10% buffered formalin, embedded in paraffin, 4 μm were sections prepared, and haematoxylin and eosin (H&E) staining was carried out using standard procedures. The Sirius stain for newly produced collagen tissue and van Giesson (VG) for connective tissue and muscle tissue was used. The Giemsa stain for highlighting of chromatin, lymphoma cells and _H. pylori_ was applied in a routine fashion. The degree of inflammation were scored from 0 to 3 in the stomach and duodenum (16). A new classification of gastric lymphoma has been published (20) and was used in this study.

Lymphoma tissues were subjected to immunohistochemistry with markers for T-cells (CD3) (CE-DARLANE® Laboratories Limited, Hornby, Ontatio, Canada, dilution 1:20) in an avidin-biotin system as well as antibody against B-cell tissue CD45R (CE-DARLANE, dilution 1:300). To outline presence of _H. pylori_ H&E, Giemsa and immunostain were carried out. The tissues were probed with an anti-H. pylori IgG fraction (dilution 1:100) developed using a Streptavidin-biotin-HRP-DAB system (DAKO, Copenhagen, Denmark) in a Tech Mate 500 staining apparatus. The grading of gastritis was evaluated evaluated according to Dixon and coworkers (21).
Immunoblot

Immunoblot was done on mice sera using an acid glycine extract of *H. pylori* NCTC 11637 as antigen, as previously described (22).

RESULTS

**Histopathology**

Gastric colonization by *H. pylori* in both C57BL/6 and BALB/cA mice inoculated with different strains of *H. pylori* at 2 weeks, 10 weeks and 23 months p.i. is shown in Table 1. The control animals, inoculated with PBS only, showed no colonization of *H. pylori* in their stomachs. Strain G50 was reisolated from mice only at 2 weeks p.i. There was no difference in colonization between strain 119p and SS1 at 10 weeks p.i., whereas SS1 gave 100% colonization versus strain 119 50% colonization in C57BL/6 mice and 0% in Balb/cA mice 23 months p.i. Interestingly, the inflammation score was higher in mice infected with strain 119p than with SS1 10 weeks p.i. (Table 2), and lymphoepithelial lesions (LEL) were found in mice infected with strain 119p and G50 but not with strain SS1 at 23 months p.i. One mouse infected with strain 119p developed primary hepatocellular carcinoma (HCC) (Table 3). No lymphoma (grade 0) was recorded in the control group.

The control animals demonstrated a well-developed corpus mucosa with specialized cells and normal foveolar surface (Fig. 1). The canal was normal, covered with foveolar cells, and no inflammatory cell reaction was noticed. In the proximal part of the gastric tissue the mucosa was covered by squamous epithelium. At the inflow area of the oesophagus into the gastric area, papillomatous-like tissue covered by squamous epithelium was seen. In that area normally a slight increase in lymphoplasmocytic cells is noted in the stroma. Slides from liver, spleen and kidney showed a normal picture without any lymphocytic or abnormal cell infiltration.

Animals infected by *H. pylori* for 23 months demonstrated a completely different feature (Fig. 2). In the junctional area between squamous epithelium and corpus mucosa, atypical glandular proliferation was also demonstrated, but no clear-cut transformation into adenocarcinoma (Fig. 2A). The lymphoma grew diffusely
TWO-YEAR FOLLOW-UP OF \textit{HELICOBACTER PYLORI} INFECTION

\textbf{TABLE 2. Inflammation scores of mouse gastritis 10 weeks post-inoculation}

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Groups</th>
<th>Body</th>
<th>Antrum</th>
<th>Duodenum</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Control (n=4)</td>
<td>0.38±0.24</td>
<td>0.88±0.13</td>
<td>0.75±0.14</td>
<td>0.67±0.17</td>
</tr>
<tr>
<td></td>
<td>SS1 (n=3)</td>
<td>1.5±0.24</td>
<td>1.5±0</td>
<td>1±0</td>
<td>1.33±0.24</td>
</tr>
<tr>
<td></td>
<td>119p (n=2)</td>
<td>3.25±0</td>
<td>3±0</td>
<td>1.5±0.35</td>
<td>2.58±0.35</td>
</tr>
<tr>
<td></td>
<td>G50 (n=2)</td>
<td>2.5±0.35</td>
<td>2±0</td>
<td>1.5±0.35</td>
<td>2±0.35</td>
</tr>
<tr>
<td>Balb/cA</td>
<td>Control (n=4)</td>
<td>0.38±0.24</td>
<td>1.13±0.13</td>
<td>0.75±0.14</td>
<td>0.75±0.17</td>
</tr>
<tr>
<td></td>
<td>SS1 (n=3)</td>
<td>1.83±0.14</td>
<td>2±0</td>
<td>1.33±0.14</td>
<td>1.72±0.14</td>
</tr>
<tr>
<td></td>
<td>119p (n=4)</td>
<td>2±0.18</td>
<td>2.25±0.22</td>
<td>1.38±0.21</td>
<td>1.88±0.20</td>
</tr>
</tbody>
</table>

\textbf{TABLE 3. Histopathology results of mice at 23 months of \textit{H. pylori} infection}

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Groups</th>
<th>Number of positive cases of HLO$^a$</th>
<th>Inflammation scores</th>
<th>Number of cases of lymphoma</th>
<th>Number of cases of high-grade lymphoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Control (n=4)</td>
<td>0</td>
<td>1.21±0.14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>SS1 (n=2)</td>
<td>2</td>
<td>1.5±0.35</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>119p (n=4)$^b$</td>
<td>3</td>
<td>2.33±0.14</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>G50 (n=3)</td>
<td>2</td>
<td>2.22±0.24</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Balb/cA</td>
<td>Control (n=4)</td>
<td>0</td>
<td>1.67±0.12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>SS1 (n=5)</td>
<td>5</td>
<td>2.77±0.13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>119p (n=4)</td>
<td>3</td>
<td>2.25±0.14</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$: HLO=\textit{Helicobacter}-like organism, diagnosed by immunostain or Giemsa stain in gastric biopsies.

$^b$: One case of hepatocellular carcinoma from this group.

DISCUSSION

in both the squamous covered mucosa, the corpus mucosa, and infiltrating deep into the ventricular wall. The tumour was also seen growing outside the gastric tissue partly surrounding the oesophagus-gastric junction. Spleen, liver and local lymph glands were also involved (2D,E,F).

In the squamous area, a squamous papillomatous proliferation was seen with hyper- and parakeratosis, achantosis and down-bulging squamous epithelium. Seemingly free floating squamous cellular areas were noted. These squamous cell areas were seen bulging into the LMM, as well as situated on the wrong side of the LMM (Fig. 2C), indicative of an early invasive, highly differentiated squamous carcinoma. Infiltrates of lymphoblastic cells were also noted in lymphoglands, liver, spleen and kidney (Fig. 2D-F). The infiltration consisted of highly abnormal lymphocytes. These cells demonstrated rough chromatin, abundant mitosis, and uneven nuclear and cell borders, and often several nuclei were noted (Fig. 2G). These were subjected to immunohistopathology with markers for T-cell (CD3) (Fig. 2H) as well as with antibody against B-cell tissue CD45R (Fig. 2I). The T-cell reactivity was basically negative while the B-cell marker was strongly positive.

One mouse, infected with strain 119p, developed primary HCC. This was mainly focally outlined by normal liver tissue in between the islands of malignant tumour cells (Fig. 3A). Heavy sclerosis was also noted, especially in the portal zones (Fig. 3B). The tumour cells were large, with rich cytoplasm and uneven nuclei, sometimes in the form of giant cells (Fig. 3C & D). Examples of mitosis, even atypical mitosis, were found. Few \textit{H. pylori} bacteria were seen inside the hepatoma tissue (Fig. 3E). H&E and Giemsa stain for gastric tissue as well as antibodies for \textit{H. pylori} showed \textit{H. pylori} in the mucus and in some glands (Fig. 3F).

Infected animals reacted with antibodies against low molecular weight antigens (26–33 kDa), and two mice infected with the SS1 strain reacted against the 120 kDa CagA toxin. Uninfected mice did not show a positive immunoblot reaction.

In our mouse model we previously described proliferative squamous epithelium with pushing borders against LMM (14). In this investigation,
Fig. 1. DCs maturation state in tolerance and immunity. The involvement of DCs in immunity and tolerance seems more complex than claimed by the bimodal model. It is evident that high levels of MHC class II and co-stimulatory molecules are not enough to induce T cell immunity. A new subset of "semi-mature" DCs, which are tolerogenic, in that they induce Treg cells, cannot be distinguished from mature DCs by their surface markers. They express high MHC class II levels and co-stimulatory molecules. Though, in the absence of microbial stimulation these semi-mature DCs do not produce pro-inflammatory cytokines. Semi-mature DCs are induced e.g. by TNF-α, apoptotic cells or the gut flora. T cell priming requires fully mature DCs, which in addition to high MHC class II and co-stimulatory molecule expression, release pro-inflammatory cytokines such as IL-12, IL-6, TNF-α and IL-1β. The fully mature DC state can be triggered by e.g. LPS, CpG or CD40L via Toll-like receptors. Immature DCs may induce T cell anergy by their low expression of MHC class II and co-stimulatory molecules and lack of pro-inflammatory cytokine release. The induction of anergy might be due to the influence of non-inflammatory cytokines as IL-10 and TGF-β.

Some squamous complexes on the wrong side of the LMM were seen, speaking in favour of early squamous cell carcinoma in this chronic model. Partly atypical glandular structures in the junction between the squamous epithelium and corpus mucosa were found. An accumulation of lymphoid tissue in the stomach is a precursor state for development of LEL of the gastric mucosa, low- and high-grade MALT lymphoma. These lesions are strongly associated with the presence of *H. pylori* infection (23–26). The MALT lymphoma formation and growth is probably antigen driven, and it has been suggested that *H. pylori* could serve as such, and proliferation of B cells could be dependent on cognate help from *H. pylori*-specific T-cells (23–26). B and T cells are recruited to the gastric mucosa as part of the complex immune response to *H. pylori*. Conversion to high-grade lymphoma might not require the presence of *H. pylori* (23, 24, 27). The histopathological features of low-grade primary gastric lymphoma resemble structures of Peyer’s patches (20, 28). Transformation of low-grade MALT-lymphoma to high-grade primary gastric lymphoma is well recognized and often a mixture of both can be found (29, 30). Sometimes only a high-grade primary gastric lymphoma is found and a de novo lymphoma formation cannot be ruled out (24). In this study, it was possible to demonstrate proliferation of the lymphoid tissue to a high-grade lymphoma state spreading to internal organs and lymph glands. As in our previous study we found only mice with high-grade lymphoma, but none with MALT-lymphoma in C57BL/6 mice.

The vacA+cagA+ strains 119p and SSI differed in their ability to colonize mouse stomach 23 months p.i. (50% and 100%, respectively). Interestingly, strain 119p induced more severe inflammation than did strain SSI, and HCC in one mouse. Only two mice infected with strain SSI and none with strain 119p reacted with antibodies to the cagA toxin (120 kDa). All reacted with antibodies to low molecular weight antigens. This emphasizes that other strain characteristics than vacA and cagA determine the severity of infection and malignant transformation. CagA has been proposed to induce a more severe disease with ulcer formation and/ or malignant transformation, but this is a matter of debate (18, 31–33).

More than 20 *Helicobacter* species are now recognized, some of which lead to various degrees of gastritis in animals. Although *H. pylori* may be the most common cause of gastrointestinal MALT lymphoma, it is not the only causative organism. *H. heilmannii* (34), other non-*H. pylori* bacteria (35) and some protozoa (36) have been observed in gastric lymphomas specific to involved regions. Animal experiments have revealed lymphoma induction, such as life-long in-
Infection of the stomach of Balb/c mice with *H. felis* resulting in gastric MALT lymphoma (27, 28). There were both gastric lymphoid tissue and glandular hyperplastic lesions found in Swiss mice with 13 months’ *H. felis* infection (38).

In addition to gastric diseases, at least 13 species colonize the lower gastrointestinal tract of domestic and laboratory animals. Several can grow in the presence of bile, and appear to be correlated with premalignant and malignant forms of liver and biliary tract disease, such as primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC) and HCC (39, 40). Recent studies on the pathogenesis of hepatic carcinoma in A/JCr mice showed that chemical carcinogens enhanced tumour development but there was no evidence of mutations in the *ras* oncogenes or the *p53* gene (41). The hepatocytes produced increased amounts of superoxide, which suggests an important role for reactive oxygen metabolites (ROM). This is analogous to the *H. pylori*-induced gastric neoplasia in chronic gastritis with a massive production of ROM by the gastric epithelium as well as by professional macrophages.

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**Fig. 2A.** Junctional area with squamous proliferation, proliferation of adenomatous glands (arrowhead) and at the top lymphoma development. HE ×5.

**Fig. 2B.** Part of the lymphomatous tissue with ulceration and to the left an island of squamous free-floating cells (arrowhead). HE ×5.

**Fig. 2C.** Squamous cell island on the wrong side of the lamina muscularis mucosa (arrowhead). VG ×5.

**Fig. 2D.** Lymphoma infiltration of the liver. HE ×5.

**Fig. 2E.** Lymphoma infiltration in the spleen. HE ×5.

**Fig. 2F.** Lymphoma in a lymph gland (arrowhead) beside normal pancreatic tissue. HE ×5.

**Fig. 2G.** Higher magnification of the lymphomatous tissue with polymorphic features of the nuclei, coarse chromatin and several examples of mitosis. HE ×100.

**Fig. 2H.** Immunohistopathological staining for CD3 (T-lymphocytes). Negative staining ×5.

**Fig. 2I.** Immunohistopathological staining for CD45R (B-lymphocytes). Negative staining ×5.
Fig. 3A. Islands of primary hepatocellular carcinoma intermingled with normal liver tissue. VG ×5.

Fig. 3B. Sirius stain of the sclerotic part of the liver tissue demonstrating abundant collagen tissue and some bile canaliculi. ×5.

Fig. 3C & 3D. Higher magnification of the hepatocellular carcinoma tissue with abnormal hepatocytes, giant cells and mitosis. HE ×40.

Fig. 3E. Immunohistopathological stain for *H. pylori* demonstrating bacteria within the hepatocellular carcinoma tissue (arrow). ×40.

Fig. 3F. Giemsa staining for *H. pylori* within gastric tissue and mucin (arrows). ×40.

(42). Whether strains of *H. pylori* and other *Helicobacter* species share common carcinogenic determinants should be explored in vitro and in animal models.

Biliary obstruction is associated with suppressed Kupffer cell clearance of bacteria and intracellular bactericidal activity of the phagocytes (43). This might facilitate the spread of *Helicobacter* spp. and their subsequent survival within the liver. In a recent overview, Fox and coworkers (39) stated that *Helicobacter* or fragments of *Helicobacter* could be found in livers of patients with PSC as well as with PBC. In addition to *H. pylori*, both *H. hepaticus* and *H. bilis* were detected. Morphologically intact bacteria were found in one patient with PSC both by immunostaining for *H. pylori* and electron microscopy (44). It was reported from France that *Helicobacter* species DNA was present in liver tissue of patients with primary HCC and from all eight control individuals, a finding which might lead to difficulties in interpretation as regards results from Hp-exposed animals (45). In Sweden, *Helicobacter* spp. were detected in patients with PBC and primary HCC but not in patients with liver metastases from colorectal carcinoma (46). *Helicobacter* organisms were recently cultured from one patient with Wilson’s disease with cirrhosis and cholestasis with a positive culture and detailed characterization (47).

In this study, we showed the development of primary HCC in one of the animals. The histopathological picture was very dramatic with enlarged atypical hepatocytes and sclerosing cholangitis around the gall canaliculi, with spreading of the tumor into local vessels. A diffuse infiltration of the lymphoma was also seen as well as *H. pylori* in immunostains. This further illustrates that *Helicobacters* might play a role in the development of gastric carcinoma and lymphoma and also of HCC. Further studies are warranted to illustrate this possibility and whether *Helicobacter* spp. are involved in the development of malignant liver and biliary tract diseases.

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