Helicobacter pylori-Associated Gastritis in Mice is Host and Strain Specific

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The vacA and cagA geno- and phenotypes of two mouse-adapted strains of Helicobacter pylori, SS1 and SPM326, were determined. The SS1 strain, which had the cagA+ and vacA s2-m2 genotype, induced neither vacuole formation in HeLa cells nor interleukin-8 (IL-8) production in KATO III cells. In contrast, H. pylori SPM326, with the cagA+ and vacA s1b-m1 genotype, induced vacuoles as well as IL-8 production in vitro. Furthermore, a spontaneous mutant of SPM326, which produced a vacuolating cytotoxin but was not able to induce IL-8 production (SPM326/IL-8−), was detected. C57Bl/6 and BALB/c mice were infected with these three strains to investigate the colonization pattern and the effect on the immune response in vivo. The SS1 strain colonized the stomachs of all mice in large numbers which remained constant over time. Colonization with SPM326/IL-8+ and SPM326/IL-8− strains was lesser, or even absent, and decreased over time. At 5 weeks postinoculation all three H. pylori strains induced a mild increase of neutrophil count in the gastric corpus of C57Bl/6 mice, which disappeared by 12 weeks. At both 5 and 12 weeks postinoculation C57Bl/6 mice colonized with SPM326/IL-8+ showed an increased expression of major histocompatibility complex (MHC) class II antigen in the cardia which was accompanied by an increased number of T cells. C57Bl/6 mice that were infected with SS1 and SPM326/IL-8− did not show chronic inflammation. BALB/c mice colonized with SS1 and SPM326/IL-8− also showed an increase in neutrophil count at 5 weeks, which normalized again by 12 weeks postinoculation. At this time point SS1-infected mice showed inflammation in the corpus and antrum. At these sites an increased expression of MHC class II antigens and an increased number of T cells were observed. Although small lymphoid follicles were already observed 5 weeks after inoculation with SS1, their incidence as well as their number was increased at 12 weeks. These results show that inflammation induced by H. pylori depends both on the bacterial strain and the host.

Helicobacter pylori infection nearly always leads to gastritis in humans. The infection is not cleared by the gastric immune response, and after a prolonged period of time more-severe clinical symptoms, such as peptic ulcer disease, carcinoma, and lymphoma, may develop (4). Several genes of H. pylori, such as vacA and cagA, are associated with this development (5). vacA, which encodes a cytotoxin, shows mosaicism in the signal (s) sequence and the middle (m) region of the gene. In addition to the previously described s1a, s1b, and s2 alleles, recently a fourth allele, s1c, was described. The m region is now expanded to include three alleles: m1, m2a, and m2b (2, 30). Several combinations of these s and m alleles are present, but s2-m1 is not. The s1-m1 and s1-m2 genotypes are associated with production of a vacuolating cytotoxin, whereas the s2-m2 genotype is considered to be noncytotoxic (2, 20, 23).

The CagA protein is about 128 kDa in size (9), and although the function of CagA is unknown, its association with peptic ulcer disease and carcinoma makes it a useful marker for virulent strains (17, 32). cagA is part of the pathogenicity island of H. pylori (1, 8). Some of the genes in this island play a role in the induction of interleukin-8 (IL-8) production by epithelial cells (1, 8, 28). IL-8 is a potent chemoattractant and activator of neutrophils and T cells and is considered to be an important contributor to inflammation (22).

Several H. pylori strains are now used in animal models, such as the mouse. However, detailed information on geno- and/or phenotype is often not available for these strains. In this study we determined the vacA s and m genotypes of and the expression of an active cytotoxin by two mouse-adapted H. pylori strains. Furthermore, the presence of cagA was determined, as well as the ability of these H. pylori strains to induce IL-8 production in a gastric cell line. C57Bl/6 mice and BALB/c mice have previously been shown to have different inflammatory responses after infection with Helicobacter felis (21, 25). Using these two strains of mice we investigated the distribution of the H. pylori strains in the stomach and the effects of their colonization on the gastric immune response.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The H. pylori strains used in this study were SS1 (18), SPM326 (19), ATCC 43504, and 93-1184 (isolated from a patient with gastritis at the University Hospital of the Vrije Universiteit, Amsterdam, The Netherlands). Bacteria used for cytotoxin and IL-8 assays were grown in brucella broth supplemented with 5% (vol/vol) newborn calf serum (NCS) (Gibco BRL, Paisley, Scotland) at 37°C for 24 h in a microaerobic atmosphere with shaking. The bacteria that were used in animal experiments were cultured on horse blood agar plates with Dent supplement (Oxoid, Basingstoke, United Kingdom), as described (31).

Genotyping by LiPA and AFLP. DNA of H. pylori was extracted by the CTAB method (3). vacA and cagA genotypes of these strains were determined by PCR and a single-step reverse hybridization line probe assay (LiPA), as described previously (29, 30). In addition, SS1, SPM326, and a spontaneous mutant of the latter strain lacking IL-8 induction capacity (SPM326/IL-8−) were analyzed by amplified fragment length polymorphism (AFLP). The AFLP was performed as described (16) with only minor modifications in the sequences of the fluoros-
cence-labeled Eco-O primer (5'-GACTGGCTACCAATTC-3') and the unla-
abeled Mse-O primer (5'-ACGGATGCTCAGGTA-3').
Assay for cytotoxin production by H. pylori strains. Cytotoxin production was
determined as described (12). Bacterial culture supernatants were filter sterilized
and stored at −20°C until use. HeLa cells (kindly provided by B. Kremer,
Academic Center for Dentistry, Amsterdam, The Netherlands) were cultured in
RPMI 1640 medium supplemented with 5% (vol/vol) NCS, 10 U of penicillin per
ml, and 10 μg of streptomycin (Gibco BRL) per ml. Cells were harvested by
trypsinization and seeded at a concentration of 3 × 10^5 cells/100 μl of tissue
culture medium in a microtiter plate. After 16 h of culture at 37°C in 95% air and
5% CO₂, cells were washed in a humidified incubator, two dilutions of bacterial culture superna-
tants in RPMI 1640 were added. After overnight incubation, the cells were
washed with RPMI 1640 and a solution of saline with 0.05% neutral red was
added. After 5 min the cells were washed with cold saline containing 0.2% NCS.
A total of 100 cells were inspected, and if more than 50 cells showed vacuoles the
H. pylori strain was designated as cytotoxin positive (12).
Assay for IL-8 induction in KATO III cells. KATO III cells (human gastric carci-
nomas cell line; kindly provided by A. van der Ende, Academic Medical
Center, Amsterdam, The Netherlands) were grown in RPMI 1640 supplemented
with 10% (vol/vol) NCS, 10 U of penicillin per ml, and 10 μg of streptomycin per
ml. The IL-8 assay was performed as described by Sharma et al. (26), with a few
modifications. Cells were seeded in 24-well tissue culture plates at 2 × 10^5 cells
well in a volume of 1 ml of tissue culture medium and were cultured for 2
days. Twelve hours before stimulation, the cells were washed with phosphate-
buffered saline (PBS), and RPMI 1640 without NCS or antibiotics was added to
the wells, to minimize the IL-8 production induced by NCS. Six to eight single
colonies of each strain were expanded, and bacteria were harvested by centri-
fugation, washed with PBS, and resuspended at a concentration of 2 × 10^7
bacteria/ml. A bacterium:cell ratio of 100:1 was used, and supernatants (50 μl) was
collected after 2 and 4 h of incubation. Surprisingly, the SS1 strain showed the s2-m2
vacuolation in HeLa cells. The other strains induced vacuola-
tion in approximately 50% of the cells. The SS1 strain was the only cagA-positive H. pylori strain that did not produce a vac-
cuolating cytotoxin.

The ability of these strains to induce IL-8 production was
assessed after 4 h of incubation. Cytotoxin-producing strains
were considered positive.

RESULTS

Genotypes and phenotypes of the H. pylori strains. The genotype
of the strains used in this study was determined by LiPA and are summarized in Table 1. The strains 93-1184 and
SS1, which showed the s2-m2 vacA genotype, did not induce
vacuolation in HeLa cells. The other strains induced vacuola-
tion in approximately 50% of the cells. The SS1 strain was the only cagA-positive H. pylori strain that did not produce a vac-
cuolating cytotoxin.

Some strains that originated from the wild-type strain, SPM326/
IL-8, were selected as negative controls.

Inoculation of mice with H. pylori. Female 6-week-old specific-pathogen-free
C57Bl/6 and BALB/c mice (Harlan CPB, Zeist, The Netherlands) were housed
under conventional conditions in our animal facilities. The mice had free access
to food and water. The Animal Care Committee of the Vrije Universiteit of
Amsterdam approved all animal experimentation described. The inoculation
with bacteria was done as described previously (18, 19). The mice received
bacteria three times in a 5-day period. A volume of 0.15 ml of bacterial suspen-
sion containing approximately 10^9 CFU/ml in PBS was given orally with a feeding
spoon. The stomach tissue was gently rubbed over the surface of freshly prepared blood
agar plates (31) containing Dent supplement (Oxoid) and 75 mg of bacitracin per
liter. After 3- to 5-day incubation at 37°C in a microaerobic atmosphere, the
numbers of H. pylori colonies present on the plates were counted. H. pylori was
identified by Gram's stain and detection of specific reactions in urease, oxidase,
and catalase tests. The presence of H. pylori in Giemsa-stained sections was also
determined microscopically.

The second experiment was conducted to determine the colonization patterns of the
different H. pylori strains in the stomach. At 4 weeks postinoculation the stomachs of 12 C57Bl/6 mice (4 mice per H. pylori strain) were divided horizon-
tally into three parts: the small zone between the nonglandular and glandular
parts of the stomach, the corpus, and the antrum. Culture of bacteria and
counting of colonies were performed as described above.

Immunohistochemistry. Longitudinal sections (thickness, 8 μm) of the stom-
ach that contained mucosal tissue from the nonglandular part of the stomach,cardia, corpus, and antrum were picked up on gelatin-coated slides, air dried, and
fixed in pure acetone for 10 min. A two-step immunoperoxidase method was
used as described previously (31). Consecutive sections were stained with mono-
clonal antibodies Ra3-6B2 (which recognizes II220 on B cells), 59-AD-22 (which
recognizes Thy-1 on T cells), and M5.114 (which recognizes major histocompati-
ibility complex [MHC] class II antigens) (31). These monoclonal antibodies were
raised in our laboratory from hybridoma cell lines. At least three sections per
mouse were investigated for the presence of inflammatory cells. Sections of the
stomach were then examined with a and were used as positive controls. Neutrophils
were recognized by staining of endogenous peroxidase.

The stomach was fixed in pure acetone for 10 min. A two-step immunoperoxidase
method was used as described previously (31). Consecutive sections were stained with mono-
clonal antibodies Ra3-6B2 (which recognizes II220 on B cells), 59-AD-22 (which
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raised in our laboratory from hybridoma cell lines. At least three sections per
mouse were investigated for the presence of inflammatory cells. Sections of the
stomach were then examined with a
Colonization of C57Bl/6 and BALB/c mice with three *H. pylori* strains. The total numbers of bacteria present in the stomachs of C57Bl/6 and BALB/c mice were determined as part of a study in which the immune response was investigated. The greatest number of colonies was observed in mice inoculated with the SS1 strain, which colonized all C57Bl/6 and BALB/c mice (Table 2). No difference in bacterial density was observed between these two mouse strains during the 12-week period of the experiment. The SPM326/IL-8*-infected C57Bl/6 mice showed an equal distribution of bacteria over the stomachs of C57Bl/6 mice. In C57Bl/6 mice infected with SPM326/IL-8* an increase of MHC class II antigen expression was increased. In control mice MHC class II antigen expression was increased only by small cells, which were located between the gastric glands, whereas in SPM326/IL-8*-infected mice MHC class II antigen expression was extended to the cells of the glands. B cells or lymphoid follicles were absent. After 12 weeks the number of neutrophils had normalized in all infected C57Bl/6 mice. In C57Bl/6 mice infected with SPM326/IL-8* the neutrophil response decreased over time in a way similar to the decrease observed in C57Bl/6 mice. One of eight SS1-infected BALB/c mice showed a follicle in the cardia at 5 weeks postinoculation. However, in some SS1-infected mice the neutrophils not only were present in the submucosa but also extended to the upper part of the corpus mucosa. In SPM326/IL-8*-infected BALB/c mice the neutrophils present in increased numbers were located in the antrum. The neutrophil response decreased over time in a way similar to the decrease observed in C57Bl/6 mice. One of eight SS1-infected BALB/c mice showed a follicle in the cardia at 5 weeks. At 12 weeks postinoculation, areas with increased MHC class II antigen expression were present in both the corpus and the antrum of SS1-colonized BALB/c mice (Fig. 3A). At these locations increased numbers of T cells were present (Fig. 3B). The number of SS1-infected mice with lymphoid follicles increased from one mouse (of eight mice) at 5 weeks to five mice (of 8 mice) at 12 weeks postinoculation. These small lymphoid follicles were observed in the deep mucosa of the cardia and occasionally also in the corpus (Fig. 3C), whereas these structures were absent in control mice. Most follicles consisted primarily of B cells. Chronic inflammation was not observed in SPM326/IL-8*-infected BALB/c mice.

During the 12-week period of infection mast cell numbers were comparable in infected and control mice (results not shown). No vacuolation of epithelial cells or ulceration was observed in the mice. Edema was occasionally observed in infected mice of both strains (Fig. 3C).

**Table 2. Colonization of C57Bl/6 and BALB/c mice by three *H. pylori* strains**

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th><em>H. pylori</em> strain (no. of inoculated mice)</th>
<th>Wks postinoculation</th>
<th>No. of mice positive for <em>H. pylori</em> (total no. of mice)</th>
<th>Log CFU/stomach*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6</td>
<td>SS1 (16)</td>
<td>5</td>
<td>8/8</td>
<td>4.17 ± 0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>8/8</td>
<td>NA*</td>
</tr>
<tr>
<td></td>
<td>SPM326/IL-8* (16)</td>
<td>5</td>
<td>7/8</td>
<td>2.64 ± 0.58†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>2/8</td>
<td>1.32 ± 0.76*</td>
</tr>
<tr>
<td></td>
<td>SPM326/IL-8* (16)</td>
<td>5</td>
<td>6/8</td>
<td>3.75 ± 0.18‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>2/8</td>
<td>3.71 ± 0.18‡</td>
</tr>
<tr>
<td>BALB/c</td>
<td>SS1 (16)</td>
<td>5</td>
<td>8/8</td>
<td>4.21 ± 0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>8/8</td>
<td>3.98 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>SPM326/IL-8* (16)</td>
<td>5</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>SPM326/IL-8* (16)</td>
<td>5</td>
<td>3/8</td>
<td>3.87 ± 0.03‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>5/8</td>
<td>2.81 ± 0.14*</td>
</tr>
</tbody>
</table>

a NA, not available. *H. pylori* was detected for eight of eight mice in Giemsa-stained sections.

* †, P < 0.009, SS1 infection compared with SPM326/IL-8* and SPM326/IL-8* at the same time point in the same mouse strain. *+, P < 0.03, infection at 5 weeks compared with at 12 weeks; †, P ≤ 0.001, infection with SPM326/IL-8* compared with SPM326/IL-8*.

**Table 3. Distribution of *H. pylori* in the cardia, corpus, and antrum of C57Bl/6 mice after 4 weeks of infection**

<table>
<thead>
<tr>
<th>No. of colonies</th>
<th>SS1 (n = 4)</th>
<th>SPM326/IL-8* (n = 4)</th>
<th>SPM326/IL-8* (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>C</td>
<td>A</td>
<td>Ca</td>
</tr>
<tr>
<td>1 × 10^2</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>10^2–10^3</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>10^3–10^4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

* Three of 4 mice were colonized.

* Ca, cardia equivalent (every part of the stomach was cultured separately); C, corpus; and A, antrum.
DISCUSSION

In this study the \textit{vacA} and \textit{cagA} genotypes of two mouse-adapted \textit{H. pylori} strains as well as the phenotypic expression of these genes were determined. C57Bl/6 and BALB/c mice were infected with these strains, which differed in cytotoxin expression and ability to induce IL-8 production, and the patterns of colonization and gastric immune response were compared.

The induction of vacuoles in HeLa cells by \textit{H. pylori} SPM326, which has \textit{vacA} genotype s1b-m1, and the lack of vacuolization by the \textit{vacA} s2-m2-possessing strain SS1 are consistent with the data of other studies (2, 20). However, recently it has become clear that the ability of the m1 or m2 cytotoxin to induce vacuoles strongly depends on the cell line that is used in the assay (20, 22). Whether \textit{H. pylori} strains with the s2-m2 genotype are able to induce vacuoles in other cell lines remains to be established.

The presence of \textit{cagA} is associated with IL-8 production by epithelial cells (10). Although both mouse-adapted strains were \textit{cagA} positive, only SPM326 induced a significantly higher level of production of IL-8 in KATO III cells compared with a \textit{cagA}-negative \textit{H. pylori} strain. In addition, a spontaneous mutant of SPM326 which induced vacuolization but did not induce IL-8 production (SPM326/IL-8-) was isolated. It has been shown that mutations in several genes of the \textit{cag} pathogenicity island affect IL-8 production by epithelial cells (1, 8, 28). Whether one of these genes is mutated in the SS1 and SPM326/IL-8- strains is currently under investigation.

Whether these three \textit{H. pylori} strains with different cytotoxin and IL-8 induction phenotypes also showed different properties in vivo was investigated in C57Bl/6 and BALB/c mice. Culture of \textit{H. pylori} from gastric biopsy specimens was found to be more sensitive to assess colonization than analysis of Giemsa-stained sections by microscopy; this result is in agreement with data reported by Shomer et al. (27). The SS1 strain showed the greatest number of colonies in culture ($10^4$ to $10^5$ CFU/stomach) and was easily detected in Giemsa-stained sections, whereas both SPM326 strains showed fewer colony numbers and were hardly detectable in sections. The detection limit for \textit{H. pylori} in sections was approximately $10^3$ bacteria/mouse stomach. The SS1 strain colonized both mouse strains in large numbers, whereas SPM326/IL-8$^+$ and SPM326/IL-8$^-$ colonized in smaller numbers and SPM326/IL-8$^-$ was not able to colonize BALB/c mice at all. Although SPM326 has been reported to show a constant colonization rate in CD1 mice for up to 52 weeks (14), we observed a decrease in the numbers of SPM326/IL-8$^-$ and SPM326/IL-8$^-$-colonized C57Bl/6 mice as well as in bacterial density over time. These results suggest that
the SPM326 strain is more susceptible to host factors than the SS1 strain.

The distribution of *H. pylori* in the mouse stomach was assessed to investigate whether colonization correlated with the gastric immune response. In both C57Bl/6 and BALB/c mice the number of neutrophils was increased at 5 weeks but not at 12 weeks postinoculation. The presence of neutrophils in the corpus or antrum was associated neither with the highest density of bacteria nor with the IL-8 induction phenotype of the colonizing *H. pylori* strain. This may not be very surprising, because mice do not express a homologue of human IL-8 (7). However, these results show that activation of neutrophils in mice occurs via mechanisms other than those observed in humans.

In contrast with the active inflammation, the presence of chronic inflammation was correlated with the highest bacterial density. In SS1-infected BALB/c mice chronic inflammation was present in the cardia, corpus, and antrum, whereas in SPM326/IL-8"-infected C57Bl/6 mice chronic inflammation was restricted to the cardia. Although SS1-infected BALB/c mice showed a density of bacteria similar to that for SS1-infected C57Bl/6 mice, chronic inflammation was absent in the last group. These observations are not in agreement with those reported by Lee et al. (18), who observed the opposite phenomenon: chronic inflammation was present at an earlier time.

FIG. 3. SS1-infected BALB/c mouse at 12 weeks postinoculation. (A) A moderate increase in MHC class II expression is observed in the upper mucosa (dark staining). (B) At the same location an increased number of T cells is present. (C) A few B cells indicate the location of a small lymphoid follicle in the deep mucosa (arrow). Note the presence of edema in the submucosa (S). M, mucosa. Bar = 20 μm.
point in SS1-infected C57Bl/6 mice than in infected BALB/c mice. This discrepancy could be due to the higher level of colonization with SS1 in C57Bl/6 mice reported by Lee et al. (18).

The chronic inflammation in the cardia of SPM326/IL-8- infected C57Bl/6 and BALB/c mice makes this mutant an inter-

ference strain that could be used to unravel the mechanism of protection against reinfection with H. pylori. Infect. Immun. 65:3222–3229.


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