Infection with cagA- and vacA-positive and -negative strains of *Helicobacter pylori* in a mouse model

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Abstract

To study the role of cytotoxin-associated protein (cagA) and vacuolating cytotoxin (vacA) in *Helicobacter pylori* infection in an experimental murine model, mice were infected with seven strains with different cagA and vacA status. Groups of 10 NMRI mice were challenged and were killed 5 weeks later. In a second study, 20 mice were challenged with a mixture of the same seven strains and killed 1, 3, 15 and 17 weeks post-inoculation. All seven strains were found to colonize the mice for the 5-week experimental period. Animals infected with vacA-positive strains, regardless of cagA status, showed an elevation of antibody titers. Two cagA-negative and vacA-positive strains and one cagA- and vacA-positive strain were found to 'take over' in the mixed infection as analyzed by the randomly amplified polymorphic DNA-polymerase chain reaction technique and in one mouse stomach we found coexistence of two of the strains. We found no evidence of the different strains colonizing different parts of the stomach. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Helicobacter pylori*; Animal model; Mouse model; Cytotoxin-associated protein; Vacuolating cytotoxin; Randomly amplified polymorphic DNA-polymerase chain reaction

1. Introduction

*Helicobacter pylori* infection causes type B gastritis in humans and is strongly associated with peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma [1–5]. It is likely that various sets of virulence factors in strains of *H. pylori* play an important role for the type of illness caused [6].

Virulence factors such as urease, the sheathed flagella, vacuolating cytotoxin (vacA), and the cytotoxin-associated protein (cagA) have been identified [7–9]. Essentially all strains of *H. pylori* have the vacA gene but the sequence and level of expression of the gene differ greatly [10,11]. CagA, on the other hand, is not present in all strains and can be used as a marker for the *H. pylori* pathogenicity island [12]. CagA and vacA are coexpressed in most, but not all cases [13]. In order to define the virulence of a strain of *H. pylori*, the expression and typing of the vacA gene and the cagA gene are commonly analyzed by Western immunoblot (WB) and PCR. In order to investigate the role of virulence factors in different diseases caused by *H. pylori* it is important to establish a small animal model to study well defined strains expressing specific virulence factors.

We have studied: (i) how *H. pylori* strains of different cagA and vacA status colonize NMRI mice and (ii) the host response to these strains.

In a second experiment we investigated: (i) if two or more strains may coexist in the stomach, (ii) whether certain strains can outgrow other strains, and (iii) whether different strains colonize different parts of the stomach.

2. Materials and methods

2.1. Animals

In two experiments 112 NMRI mice were kept on a 12-h night/day schedule and fed the RM2 diet (B&K Universal, Sollentuna, Sweden) and water ad libitum [14]. The experiments were approved by Lund University’s ethical committee, permit no. M102-97.
2.2. Bacteria

From an in-house collection of clinical isolates and reference strains, cagA and vacA genotypes and expression were determined. Eight strains were chosen, two strains representing each of four different subtypes (Table 1). Six of these were clinical isolates from Lund University Hospital, Sweden; a seventh strain (G50) was kindly donated by Dr. T. Boreén and the eighth strain (SS1), a strain known to readily colonize mice, was kindly donated by Dr. A. Lee [15]. Prior to inoculation all strains were cultured in tryptose soy broth with 10% horse serum (TSB) for 48 h, as described by McColm et al. [16].

2.3. CagA and vacA expression

The expression of cagA and vacA was analyzed by WB as described below. A total protein extract from each of the strains was obtained by 10 cycles of ultrasonication at 35 kHz for 30 s with 20-s intervals between cycles. The protein content in each extract was determined by Coo massie brilliant blue G-250 dye assay (Bio-Rad, Richmond, CA, USA).

SDS-PAGE was performed under reducing conditions in a Protean II slab gel vertical electrophoresis machine (Bio-Rad) using a 5–20% separating gradient gel [17]. Polypeptides resolved by SDS-PAGE were electrophoretically transferred to PVDF membranes (Micron Separations Inc., Westborough, MA, USA), using a semi-dry electrobetter. Immunoblotting was performed as previously described using an antiserum specific to vacA (obtained from Dr. D. Burroni, Chiron Vaccines, Siena, Italy) and cagA (A.F. Schützdeller Biochemica, Tübingen, Germany) [18].

2.4. Experiment 1

Seven groups of 10 NMRI mice were challenged twice within 3 days with 1 ml of a 48-h TSB culture of the mouse-passaged strains. The concentration of the inoculum ranged from $6.0 \times 10^6$ to $1.8 \times 10^8$ CFU ml$^{-1}$ (Table 2). Ten animals were challenged twice with 1 ml sterile TSB and used as control. All animals were killed 5 weeks post-inoculation.

2.5. Experiment 2

The seven strains used in experiment 1 were cultured for 48 h, mixed and used to infect 20 NMRI mice once with 1 ml each. Prior to mixing, the cultures were diluted to the same optical density to ensure similar cell concentrations. Dilution series for each of the strains showed concentrations ranging from $3.2 \times 10^6$ to $1.4 \times 10^7$ CFU ml$^{-1}$ except for strain 28/97 which had a concentration of $2.3 \times 10^4$ CFU ml$^{-1}$ (Table 2). As controls, 12 mice were inoculated with 1 ml of sterile TSB each. Five challenged and three control animals were killed at 1, 3, 15, and 17 weeks post-inoculation.

2.6. Sample collection

Mice were killed using CO$_2$, blood was collected by heart puncture, and sera were frozen at $-20^\circ$C for serology. The stomach was removed, opened along the major curvature, rinsed in sterile phosphate-buffered saline, pH 7.2, and divided along the midline. One half of the stomach was used for culture on Gab-Camp agar [19]. The mucosa of the corpus region in mice is thicker and has a distinctly darker color than the antrum region making it possible to identify the borderline visually. This was used in the second experiment where each stomach half used for culture was divided at the borderline between the corpus

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### Table 1

<table>
<thead>
<tr>
<th>H. pylori strain</th>
<th>CagA</th>
<th>VacA</th>
<th>WB PCR</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>119/95</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>s1</td>
</tr>
<tr>
<td>SS1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>s2</td>
</tr>
<tr>
<td>114/97</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1</td>
</tr>
<tr>
<td>28/97</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>s1</td>
</tr>
<tr>
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<td>-</td>
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<td>s1</td>
</tr>
<tr>
<td>15/96</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>s2</td>
</tr>
</tbody>
</table>

*Signal sequence allele.

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### Table 2

<table>
<thead>
<tr>
<th>H. pylori strain</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. 1st challenge (CFU ml$^{-1}$)</td>
<td>Conc. 2nd challenge (CFU ml$^{-1}$)</td>
</tr>
<tr>
<td>119/95</td>
<td>$6.0 \times 10^6$</td>
<td>$3.9 \times 10^7$</td>
</tr>
<tr>
<td>SS1</td>
<td>$1.2 \times 10^6$</td>
<td>$1.7 \times 10^6$</td>
</tr>
<tr>
<td>114/97</td>
<td>$4.8 \times 10^7$</td>
<td>$7.7 \times 10^7$</td>
</tr>
<tr>
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<td>$1.2 \times 10^7$</td>
<td>$1.8 \times 10^8$</td>
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<td>10/96</td>
<td>$5.0 \times 10^7$</td>
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</tr>
<tr>
<td>6/97</td>
<td>$7.0 \times 10^6$</td>
<td>$8.4 \times 10^7$</td>
</tr>
<tr>
<td>15/96</td>
<td>$4.8 \times 10^7$</td>
<td>$5.5 \times 10^7$</td>
</tr>
</tbody>
</table>

*Number of culture-positive mice/number of challenged mice.
and antrum regions, and each part cultured separately. The second half of the stomach was fixed in 10% buffered formaldehyde. Culture results were evaluated after 8 days of culture under microaerobic conditions at 37°C. In the second experiment eight individual colonies were, when possible, subcultured from each culture-positive plate for analysis by randomly amplified polymorphic DNA (RAPD)-PCR (see below). In order to make sure that none of the strains would take over when subcultured, only samples from primary plates, and secondary plates when necessary, were used in the RAPD-PCR analysis.

2.7. Serology

Enzyme immunoassay (EIA) and WB were done with glycine-extracted \textit{H. pylori} cell surface protein antigen as described by Guruge et al. [20].

EIA and WB were performed on mouse sera using horseradish peroxidase-labelled goat anti-mouse immunoglobulins (Dakopatts, Copenhagen, Denmark) as described by Wang et al. [21]. The EIA absorbance levels at 450 nm were measured using a spectrophotometer (Lab-system Multiscan Plus, Helsinki, Finland). From the EIA absorbance levels, a relative antibody activity (RAA) was calculated using the formula [relative antibody activity $= \frac{\text{mean absorbance}}{100} / \text{mean absorbance of reference}$], where mean absorbance is the average of the two wells run on each sample with the background subtracted and mean absorbance of reference is the average of the four reference wells [22].

2.8. Histopathology

Formaldehyde-fixed stomachs were embedded in paraffin. Sections, 4 μm thick, were prepared and stained with hematoxylin and eosin following standard procedures. The level of gastritis in the corpus and antrum regions was graded blind by two examiners from 0 to 3 using the system developed for the guinea pig model of \textit{H. pylori} infection [22]. In this grading system 0 corresponds to no gastric inflammation, 1 to mild, 2 to moderate, and 3 to severe gastric inflammation.

2.9. DNA extraction

A loopful of bacteria, collected from one colony, was suspended in 50 μl of physiological NaCl solution and mixed with an extraction buffer (75 mM KCl, 3 mM EDTA, 150 mM Tris–HCl (pH 8.0), 0.75% Tween 20) previously described by Nilsson et al. [23]. The samples were then incubated at 22°C for 10 min, vortexed and incubated at 90°C for 10 min and finally put on ice for 4 min. After centrifugation for 10 min at 12,000 × g, 100 μl of the fluid phase was recovered and used as a DNA template in RAPD-PCR.

2.10. PCR and RAPD-PCR

The presence of cagA in each strain was determined by PCR as previously described [8]. The vacA signal sequence allele (s1 or s2) was determined using vac10F and vac10R primers [24]. The PCR reaction mixture consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 0.01% bovine serum albumin, 0.2 mM of each deoxynucleotide triphosphate (Boehringer, Mannheim, Germany), 0.5 μM of each oligonucleotide primer (Scandinavian Gene Synthesis, Köping, Sweden), and 1.5 U of Taq polymerase (MBI Fermentas, Vilnius, Lithuania). 50 μl was used for the PCR reaction which was performed in a Perkin-Elmer GeneAmp 2400 thermal cycler (Perkin-Elmer, Norwalk CT, USA). Amplified products were analyzed by 1.5% agarose gel electrophoresis. The size of the amplified fragments was estimated by comparison with 100-bp DNA size markers (MBI Fermentas, Vilnius, Lithuania).

Prior to infection of the NMRI mice, each strain was analyzed by RAPD-PCR using primers 1283 and 1254 as previously described [25] (Fig. 1). RAPD-PCR was performed as described above with the following modifications: 3 mM MgCl$_2$, 2.5 U of Taq polymerase and 0.3 mM of each deoxynucleotide triphosphate were used. After challenging the mice, \textit{H. pylori} isolates from each culture-positive mouse were extracted and analyzed by RAPD-PCR.

2.11. Statistical analysis

The serum RAA levels for \textit{H. pylori}-challenged mice were compared to control animals using unpaired one-
sided Mann–Whitney U-test; \( P < 0.05 \) was considered significant.

3. Results

3.1. Culture

To ascertain the virulence of the strains, all were passaged once in NMRI mice prior to this study. One of the strains (G50: cagA-negative/vacA-negative) could not be recovered from challenged mice upon three consecutive attempts and was not used in further experiments. The seven remaining strains could all be recovered 1 week post-inoculation.

All seven strains were found to colonize NMRI mice for the 5-week experimental period in experiment 1 regardless of cagA/vacA status, but the number of culture-positive animals in each group varied markedly (Table 2).

In the second experiment gastric colonization by \( \text{H. pylori} \) was found in all five animals at 3 weeks, one of five at 15 weeks and one of five at 17 weeks post-inoculation as assessed by culture. At 1 week post-inoculation, \( \text{H. pylori} \) could not be recovered from the five challenged animals that were killed.

3.2. Histopathology

In experiment 1, nine infected mice exhibited grade 1 gastritis, of these animals four were infected with strain SS1.

One mouse in experiment 2 had grade 1 gastritis at 17 weeks post-inoculation. No gastritis was found among the other animals.

3.3. Serology

All culture-positive animals in experiment 1 showed weak bands on WB. In EIA, the average RAA was found to be elevated among culture-positive animals infected with strains SS1, 6/97, and 10/96. The average RAA among culture-positive animals in the groups infected with the other strains was not elevated, even though individual animals of each group exhibited high RAA values (see Fig. 2).

The \( \text{H. pylori} \)-challenged animals in experiment 2 killed after 15 and 17 weeks all exhibited bands on WB corresponding to 19, 25.5 and 29.5 kDa, similar to the findings after 3 and 7 weeks of \( \text{H. pylori} \) infection in guinea pigs [22]. Similarly \( \text{H. pylori} \)-challenged animals showed elevated RAA levels after 15 and 17 weeks. No control animals or animals killed at the earlier time points showed bands on this WB analysis or exhibited elevated RAA levels.

3.4. RAPD-PCR

From the five animals killed 5 weeks after inoculation, \( \text{H. pylori} \) strains detected in culture-positive NMRI mice by RAPD-PCR

<table>
<thead>
<tr>
<th>Mouse number</th>
<th>Section</th>
<th>Number of colonies analyzed</th>
<th>Strain</th>
<th>Colonization time (weeks)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Corpus</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antrum</td>
<td>8</td>
<td>10/96</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>Corpus</td>
<td>8</td>
<td>6/97</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Antrum</td>
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<td>6/97</td>
<td>5</td>
</tr>
<tr>
<td>14</td>
<td>Corpus</td>
<td>8</td>
<td>10/96</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Antrum</td>
<td>8</td>
<td>10/96</td>
<td>5</td>
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<tr>
<td>15</td>
<td>Corpus</td>
<td>8</td>
<td>10/96</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Antrum</td>
<td>8</td>
<td>10/96</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>Corpus</td>
<td>3</td>
<td>6/97</td>
<td>5</td>
</tr>
<tr>
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<td>Antrum</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Corpus</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antrum</td>
<td>6</td>
<td>SS1 (5 col.) and 6/97 (1 col.)</td>
<td>15</td>
</tr>
<tr>
<td>30</td>
<td>Corpus</td>
<td>14</td>
<td>SS1</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Antrum</td>
<td>10</td>
<td>SS1</td>
<td></td>
</tr>
</tbody>
</table>
4. Discussion

We have shown that *H. pylori* strains of different cagA and vacA status can colonize the NMRI mouse stomach (Tables 1 and 2). In a similar set-up, Sheu et al. [26] managed to infect BALB/c mice with cagA-negative strains. In our experiments mice infected with the cagA-positive/vacA-positive strain SS1 showed higher levels of gastritis than mice infected with the other strains but the level of gastritis recorded was not severe. Antibody titers were found to be elevated among animals challenged with vacA-positive strains regardless of the cagA status. *H. pylori* antibody elevation was not found among animals challenged with vacA-negative strains. The importance of vacA in the serological response is consistent with earlier findings in a mouse model [27]. CagA did not seem to promote the serological response indicating that vacA is more immunogenic in *H. pylori* murine infection than the cagA protein. Studies in a Mongolian gerbil model of *H. pylori* infection indicate a lower infection rate with cagA-negative than with cagA-positive strains [28]. However, in our present study the two strains that gave the highest colonization rate were both cagA-negative and one was also vacA-negative. Thus our data indicate that the cagA protein and vacA toxin are not crucial for *H. pylori* to colonize and infect the murine stomach, but that vacA promotes an immune response. The lack of severe gastritis in all infected animals is consistent with earlier findings in the mouse model, but perhaps more severe gastritis could be induced in other mouse strains such as the C57 black mouse [15]. If chronic active gastritis is to be studied it might be better to establish these *H. pylori* strains in either the Mongolian gerbil or the guinea pig model since both these animals develop severe gastric inflammation in response to *H. pylori* infection [22,29–31].

The results from the RAPD-PCR analysis of *H. pylori* isolates indicate that strains lacking the cagA gene can become the prominent strain in a simultaneous infection with other strains. Similar to earlier studies, we found that two strains of *H. pylori* can coexist in the murine stomach [32]. Further studies will show whether one strain can eradicate an already established strain, suggesting that it may be possible, in the future, to colonize patients with a strain of *H. pylori* of low virulence to protect against strains causing severe type B gastritis. A similar strategy is already being explored with colonization in patients with recurrent urinary tract infections with a harmless strain of *Escherichia coli* [33].

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References


