Role of Vacuolating Cytotoxin in Gastritis Due to *Helicobacter pylori* in Gnotobiotic Piglets

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To investigate the role of the *Helicobacter pylori* cytotoxin in the pathogenesis of gastritis, gnotobiotic piglets were colonized with either toxigenic *H. pylori* or a nontoxigenic isogenic mutant. Only piglets given the toxigenic strain developed toxin-neutralizing antibodies (indicating that toxin is expressed in vivo), but there was no difference in bacterial colonization, epithelial vacuolation, or gastritis between the two groups of piglets.

The discovery in 1983 of the association between *Helicobacter pylori* and peptic ulceration (17) led to intense study of potential bacterial virulence factors. One such factor is the vacuolating cytotoxin (VacA), a multimeric protein with subunits of approximately 90 kDa which induces cytoplasmic vacuolation in cultured epithelial cells (5, 6, 16). All strains of *H. pylori* contain the cytotoxin gene, vacA, but only about 50% of strains (2) produce detectable cytotoxin activity in vitro. The goal of the present study was to investigate the role of vacuolating cytotoxin in colonization, gastritis, and epithelial vacuolation due to *H. pylori* in gnotobiotic piglets.

The *H. pylori* strains used were 26695, a cytotoxin-producing human isolate with a type s1 vacA allele (3a) that had been passaged in piglets more than 10 times (11, 15), and an isogenic mutant, 26695vacAαtaph3−III, derived from the pig-passaged 26695 by natural transformation and insertional mutagenesis by previously described methods (7, 19). Bacteria were grown in a microaerobic atmosphere (5% oxygen, 10% carbon dioxide, 85% nitrogen) at 37°C on Trypticase soy agar plates containing 5% sheep’s blood. Prior to animal inoculation, bacteria were grown in brucella broth with 10% fetal calf serum.

Twenty-five piglets from three litters were derived by cesarean section and maintained in sterile isolators as previously described (15). At 3 days of age, they were inoculated orally with 10⁹ CFU of either 26695 or 26695vacAαtaph3−III and killed either 2 or 28 days after inoculation (see Table 1 for numbers of piglets per group). At necropsy, bacterial colonization was quantified by plate dilution (9). Hematoxylin-and-eosin-stained sections of gastric mucosa were coded and examined blindly (without knowledge of their source) for the presence of lymphocytic and neutrophilic inflammation on a six-point scale as follows: 0 = no inflammation; 1 = mild multifocal inflammation; 2 = mild, widespread inflammation; 3 = mild, widespread and moderate, multifocal inflammation; 4 = moderate, widespread inflammation; 5 = moderate, widespread and multifocal, severe inflammation; and 6 = severe, widespread inflammation. Epithelial vacuolation was scored on a similar six-point scale (0 = no vacuolation; 1 = mild, multifocal vacuolation; 2 = mild, widespread vacuolation; etc.). Means were compared by the Mann-Whitney U test.

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In contrast, antitoxin serum titers from the piglets given 26695vacA and killed 28 days after inoculation were all less than or equal to 1:10, and none of the piglets developed serum toxin-neutralizing activity.

All piglets in this study were infected, and all developed lymphoplasmacytic and follicular gastritis, in contrast to uninfected piglets, which do not develop gastritis (9–12, 15). At both sacrifice intervals, the mean lymphoplasmacytic gastritis score was slightly greater in piglets given 26695 than in piglets given 26695vacA and an isogenic cytotoxin-negative mutant 9 vacA V aph3 -III, but these differences did not reach statistical significance (Table 1). Neutrophilic gastric inflammation, characterized by mild, multifocal infiltrates of neutrophils in the deep lamina propria, was present only in piglets killed 2 days after inoculation. Five of nine piglets given 26695 and three of eight piglets given strain 26695vacA and killed 28 days after inoculation had neutrophilic inflammation (Table 1).

Most piglets had mild to moderate vacuolation of superficial epithelial cells, regardless of whether they were infected with strain 26695 or 26695vacA and killed 28 days after inoculation. This change was characterized by clusters of 3- to 4-μm vacuoles in the cytoplasm of the surface mucus cells (Fig. 1). Occasionally, severe foci of vacuolation were associated with cellular degeneration or necrosis of epithelial cells (Fig. 2). Epithelial vacuolation was slightly more common and severe in piglets killed 28 days after inoculation than in those killed 2 days after inoculation, but the differences were not statistically significant (Table 1).

The gnotobiotic piglet model of infection uses H. pylori of human origin and results in persistent bacterial colonization (approximately 10^6 to 10^7 CFU/g of gastric mucosa) and chronic lymphocytic and follicular gastritis (4, 11, 15). Some piglets also develop mild transient neutrophilic inflammation.

The results of this study revealed that, in piglets, the vacA gene product did not have a demonstrable role in bacterial colonization, inflammation, or epithelial vacuolation and necrosis. This was despite evidence of toxin expression in vivo as indicated by the development of serum antitoxin antibody in piglets given toxigenic, but not nontoxigenic, H. pylori. Previous studies have shown that epithelial vacuolation in H. pylori-infected piglets that in uninfected piglets (11). We speculate that the observed association between vacuolation and infection may result from the development of serotonergic neural pathways (4, 11, 15).

Several studies have suggested that H. pylori VacA might be an important factor contributing to the pathogenesis of peptic ulcer disease in humans. Isolates from patients with ulcer disease more frequently induce vacuolation of cells in vitro than do isolates from patients without ulcers, and infection with H. pylori strains containing type s1 vacA alleles is associated with an increased incidence of duodenal ulcer disease and increased severity of gastric and epithelial damage (2, 3, 8, 13). This study was not able to evaluate the effect of VacA on gastric ulceration in piglets. Only about 20% of piglets infected for 28 days or more developed epithelial ulceration (14), and in this study, none of the four piglets given 26695 and killed after 28 days developed ulcers. Thus this group could not be compared to the piglets given 26695vacA and killed 28 days after inoculation.

Of final importance, this study definitively establishes the usefulness of animal models in evaluating H. pylori bacterial virulence factors by using mutants derived by insertional mutagenesis. Previously, all mutants tested in animal models ei-

### TABLE 1. Bacterial colonization, gastritis, epithelial vacuolation, and neutrophilic inflammation in piglets colonized with H. pylori 26695 and an isogenic cytotoxin-negative mutant

<table>
<thead>
<tr>
<th>Time of sacrifice (after inoculation) and strain</th>
<th>n</th>
<th>Colonizationb (log 10 CFU/gram, mean ± standard deviation)</th>
<th>Gastritisb (lymphoplasmacytic gastritis score, shown as mean ± standard deviation)</th>
<th>No. with vacuoles</th>
<th>Vacuole score</th>
<th>No. with neutrophils</th>
<th>Neutrophil score (see the text), shown as mean ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26695</td>
<td>9</td>
<td>7.32 ± 0.34</td>
<td>2.00 ± 0.85</td>
<td>7</td>
<td>1.75 ± 1.39</td>
<td>5</td>
<td>1.33 ± 0.52</td>
</tr>
<tr>
<td>26695vacA and aph3 -III</td>
<td>8</td>
<td>7.39 ± 0.44</td>
<td>1.36 ± 0.67</td>
<td>8</td>
<td>1.36 ± 1.02</td>
<td>3</td>
<td>1.67 ± 1.15</td>
</tr>
<tr>
<td>28 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26695</td>
<td>4</td>
<td>6.99 ± 0.63</td>
<td>2.25 ± 0.87</td>
<td>4</td>
<td>2.11 ± 1.36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>26695vacA and aph3 -III</td>
<td>4</td>
<td>6.78 ± 0.94</td>
<td>1.83 ± 0.72</td>
<td>4</td>
<td>2.57 ± 1.27</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Bacterial colonization (log 10 CFU/gram, mean ± standard deviation).
b Lymphoplasmacytic gastritis score (see the text), shown as mean ± standard deviation.

* Not significantly different from 26695 group; P > 0.05.
ther failed to colonize or colonized poorly and did not persist (1, 10, 12, 18), suggesting that the bacterial virulence factors tested (flagella and urease) were essential for colonization. However, another possible interpretation would have been that the process of mutagenesis itself hindered colonization. The successful colonization by 26695_vacA_Maph3′-III in this study indicates that insertion mutagenesis is a valid approach for identifying colonization factors.

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REFERENCES


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