Roles of \textit{virD4} and \textit{cagG} genes in the \textit{cag} pathogenicity island of \textit{Helicobacter pylori} using a Mongolian gerbil model

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Background and Aims: The roles of the \textit{virD4} and \textit{cagG} genes in the \textit{cag} pathogenicity island of \textit{Helicobacter pylori} for gastroduodenal pathogenesis are unclear and their roles in vivo have not been examined.

Methods: Seven week old male Mongolian gerbils were inoculated with the wild type \textit{H pylori} TN2GF4, its isogenic \textit{virD4}, or \textit{cagG} mutants. Animals were sacrificed at 4, 12, and 24 weeks after inoculation. Gastric inflammation and \textit{H pylori} density were evaluated by histology, inflammatory response (as measured by interleukin (IL)-1β mRNA levels), proliferative activity (as assessed by 5'-bromo-2'-deoxyuridine labelling indices), and host systemic reaction (as measured by anti-\textit{H pylori} IgG antibody).

Results: Degree of gastric inflammation, proliferative activity, and mucosal IL-1β mRNA levels remained low throughout the first 12 weeks in gerbils infected with the \textit{virD4} mutants. Degree of gastric inflammation and proliferative activity increased at 24 weeks with the \textit{virD4} mutants reaching levels comparative with those seen at four weeks with the wild-type strains. Mucosal IL-1β mRNA levels were also increased at 24 weeks with the \textit{virD4} mutants and levels at 24 weeks were similar between the wild-type and \textit{virD4} mutants. In contrast, gerbils infected with the \textit{cagG} mutants had reduced ability to colonise gerbils, and no or little gastric inflammation or proliferative activity was observed.

Conclusions: Loss of the \textit{virD4} gene temporally retarded but did not abrogate gastric inflammation. Loss of the \textit{cagG} gene abolished gastric inflammation partially via reduced ability to colonise gerbils. Unknown factors related to the type IV secretion system other than CagA may influence gastric inflammation.
Roles of virD4 and cagG of H pylori in gerbils

Osaka, Japan) and its isogenic knockout mutants for cagG and virD4. Strain TN2GF4 was isolated from Japanese gastric ulcer patients and is reported to induce gastric ulcer and gastric cancer in gerbils over 62 weeks.13 Isogenic mutant strains were constructed from a single colony from stock frozen H pylori. A portion of the genes encoding the cagG and virD4 genes was amplified by polymerase chain reaction (PCR) and the amplified fragment was inserted into the EcoRV restriction enzyme site of pBluescriptSK+ (Stratagene, La Jolla, California, USA). A in triplicate.

Southern blot hybridisation. 

genes was confirmed by PCR amplification followed by BsmI inserted into the chloramphenicol resistance gene cassette (a gift from DE EcoI was inserted into the sorbent assay (R&D Systems, Minneapolis, Minnesota, USA) in triplicate.

IL-8 levels from gastric cancer cells cocultured with H pylori

In vitro IL-8 measurement was performed as previously described.23 Briefly, the human gastric cell line MKN 45 (Japanese Cancer Research Bank, Tsukuba, Japan) (1×10⁵/ml) was plated onto 24 well plates and cultured for two days. H pylori was added to the cultured cells (bacterium to cell ratio of 100:1) and incubated for 24 hours. IL-8 in the supernatant was measured by an enzyme linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota, USA) in triplicate.

Animal, housing, and H pylori challenge

Specific pathogen free seven week old male Mongolian gerbils (MGS/Sea; Seac Yoshitomi, Fukuoka, Japan) were used in this study. They were housed in an air conditioned biohazard room designed for infectious animals, with a 12 hour light/12 hour dark cycle. They were provided with a rodent diet and water ad libitum. All experimental protocols were approved by the Animal Experiment Committee of Shinshu University School of Medicine, Matsumoto, Japan. H pylori were grown in Brucella broth supplemented with 10% (vol/vol) horse serum for 40 hours at 37°C under microaerobic conditions and saturated humidity, with shaking at 150 rpm. After fasting for 24 hours, each animal was oroantrally inoculated with 1.0 ml of an inoculum prepared of H pylori (10⁶ colony forming units (CFU)/ml) or sterile Brucella broth (as an uninfected control) using gastric intubation needles. No specific pretreatments (for example, acid inhibition or antibiotics) were used before orogastric H pylori inoculation. Four hours after administration, animals were again allowed free access to water and food.

Time course and euthanasia

Mongolian gerbils were assigned to one of three groups: inoculated with the wild-type H pylori strains, with its cagG mutants, or with its virD4 mutants. Infected gerbils were killed and underwent necropsy at 4, 12, and 24 weeks after H pylori inoculation. Eight to nine gerbils were used for each time point. Uninfected control gerbils were killed at 11, 19, and 31 weeks of age (to serve as controls for the infected animals 4, 12, and 24 weeks after H pylori inoculation) (n = 6 each). Thirty minutes before being killed, gerbils were given 200 mg/kg of 5'-bromo-2'-deoxyuridine (BrdU) intraperitoneally.

At necropsy, stomachs were opened along the greater curvature, beginning at the gastro-oesophageal junction and ending at the proximal portion of the duodenum, and observed macroscopically. Stomachs were then divided longitudinally into two parts and one half was fixed in 20% phosphate buffered formalin fixative for histological examination. The other part was further divided into the pyloric gland mucosa (antrum) and the fundic gland mucosa (corpus). The gastric mucosa was separated as much as possible from the underlying muscle using sharp dissection. Each specimen was placed on dry ice and stored at −80°C for cytokines mRNA analysis.

Table 1. Primers and probes used in this study

<table>
<thead>
<tr>
<th>GAPDH</th>
<th>Primer Forward: 5'-CATGGCCTTCCGAGTCTTCC-3'</th>
<th>Probe: 5'-VIC-CGCCAAACGTCTGCTGCGTGGA-TAMURA-3'</th>
<th>IL-1β</th>
<th>Primer Forward: 5'-GGTGAACACACGACAGCAGACAA-3'</th>
<th>Reverse: 5'-CATCAACCAAGGACAGTACAGTCTTCC-3'</th>
<th>Probe: 5'-FAM-TACCGTGGCTGGCCCTCA-TAMURA-3'</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>glycereraldehyde-3-phosphate dehydrogenase; IL, interleukin; FAM, 6-carboxyfluorescein; TAMURA, 6-carboxy-N, N', N'-tetramethylrhodamine.</td>
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Histological examination

Half of the stomach was stapled onto paper and fixed in 20% phosphate buffered formalin for 24 hours at 4°C. The fixed gastric tissue was processed for histopathological examination, and paraffin embedded sections were sliced and stained with haematoxylin-eosin or May-Grünwald-Giemsa. The degree of inflammation was graded according to the updated Sydney system.24 Analysis of IL-1β mRNA expression by real time quantitative PCR

Total RNA was extracted from the gastric mucosa using an RNA extraction kit (Isogen; Nippon Gene, Tokyo, Japan). After DNase treatment, 5 μg of total RNA were subjected to reverse transcription (RT) using 200 U of Moloney murine leukaemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, Maryland, USA). Partial gerbil specific IL-1β cDNA sequences were recently cloned in our group (GenBank accession number AB164705) and we normalised IL-1β mRNA levels to the gerbil specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA identified previously.11 Specific primers and TaqMan probes are listed in table 1. Real time PCR was performed using an ABI Prism 7700 Sequence-Detection System (Perkin-Elmer Applied Biosystems) at 50°C for two minutes, 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds. IL-1β mRNA levels were expressed as the ratio of IL-1β mRNA to GAPDH mRNA (100 000 × IL-1β mRNA (unit/μl)/GAPDH mRNA (unit/μl)). Each assay was performed in triplicate.

Sero-logy

Before the animals were killed, blood samples were obtained from the orbital plexus using haematocrit tubes. Sera were used to measure the titre of anti-H pylori IgG antibody, as previously described.25 26 Antibody titre was expressed as an arbitrary index (AI) with values greater than 1.37...
H pylori cagG induced intermediate levels of IL-8 whereas the virD4 mutants showed very low (maximum 3.5), and even seroconversion occurred.

Histopathological findings
Histopathological changes at 4, 12, and 24 weeks after inoculation of Mongolian gerbils with H pylori and in controls are shown in fig 2. Inflammatory cell infiltration in the lamina propria was negligible in controls. At four weeks after inoculation, gerbils infected with the wild-type strains showed chronic active gastritis in the antrum, with marked mucosal infiltration by neutrophilic polymorphonuclear cells (PMN) (infiltration score 1.0 (0.3)) and by mononuclear cells (MNC) (1.9 (0.3)) (figs 3, 4). At 12 weeks, with the wild-type strains, dense PMN infiltration was seen throughout the mucosa with a dense MNC infiltration in the lamina propria and submucosa in the antrum, with the normal mucosal architecture being almost completely replaced with hyperplastic epithelium (PMN 2.5 (0.2) and 0.7 (0.2); MNC 2.8 (0.1) and 1.0 (0.2) for the antrum and corpus, respectively). At 24 weeks with the wild-type strains, numerous irregularly branched dilated mucous glands were seen in the lower portion of the proper muscle layer and the PMN and MNC infiltration scores reached their maximal levels (PMN 3.0 and 1.0 (0.2); MNC 3.0 and 1.1 (0.1) for the antrum and corpus, respectively).

In contrast, gerbils infected with the cagG mutants showed almost no inflammation at any time after inoculation (figs 3, 4). MNC and PMC infiltration scores in gerbils infected with the cagG mutants were significantly lower than those with the wild-type strain throughout the observation periods.

Gerbils infected with the virD4 mutants showed mild cellular inflammation four and 12 weeks after inoculation (MNC and PMC infiltration scores less than 0.5). MNC and PMC infiltration scores in gerbils infected with the virD4 mutants were significantly lower than those with the wild-type strains throughout the observation periods. Interestingly, however, at 24 weeks after inoculation, gerbils infected with the virD4 mutants showed chronic active gastritis with marked mucosal infiltration in the antrum (MNC 1.8 (0.3) and PMN 1.3 (0.3)) whereas mucosal infiltration in the corpus remained very mild (figs 3, 4). The amount of cellular infiltration in the antrum increased in gerbils infected with the virD4 mutants at 24 weeks.
compared with 12 weeks, and the pyloric mucosa appeared expanded similar to that observed with the wild-type strains. The grade of mucosal inflammation observed in gerbils infected with the virD4 mutants at 24 weeks was similar to those with the wild-type strains at four weeks.

*H. pylori* density score, as evaluated by histology in the antrum of gerbils infected with the virD4 mutants, was significantly greater than that in animals infected with the wild-type strains at 24 weeks (fig 5) (p<0.01). Importantly,
gerbils infected with the cagG mutants showed no inflammation at any time after inoculation. *H pylori* density score was mostly equivalent to the wild-type strains or virD4 mutants (fig 5).

**BrdU labelling indices**

Detectable BrdU labelling indices were not observed in control gerbils without *H pylori* infection. BrdU labelling indices in the antrum were independent of the duration of *H pylori* infection both in gerbils infected with the wild-type strains (mean 26.3 (3.1) to 34.6 (3.3)) and the cagG mutants (mean 3.2 (0.5) to 11.3 (7.3)) (fig 6). In contrast, BrdU labelling indices were significantly increased in the virD4 mutants at 24 weeks after inoculation (29.3 (3.9) at 24 weeks compared with 6.1 (0.8) at four weeks and 3.8 (0.5) at 12 weeks) (p<0.001 for each). Overall, BrdU labelling indices were higher in gerbils infected with the wild-type strains compared with those with the cagG mutants or the virD4 mutants at four or 12 weeks. Indices were also significantly higher in gerbils infected with the wild-type strains compared with those with the cagG mutants at 24 weeks (p<0.001) whereas the indices were similar among gerbils infected with the wild-type strains and the virD4 mutants.

**Mucosal IL-1β mRNA levels**

In the control group, mucosal IL-1β mRNA levels were very low throughout the observation periods (10000× mean (SEM); IL-1β/GAPDH 1.8 (0.4) to 6.6 (0.8)) (fig 7). At four weeks after inoculation, mucosal IL-1β mRNA levels were significantly greater in gerbils infected with the wild-type strains compared with the virD4 or cagG mutants. Mucosal IL-1β mRNA levels at 12 weeks with the wild-type strains were also significantly higher than those with the virD4 or cagG mutants. At 24 weeks after inoculation, IL-1β levels decreased in gerbils infected with the wild-type strains.

Mucosal IL-1β levels were very low in gerbils infected with the virD4 mutants throughout the first 12 weeks; however, these levels tended to increase at 24 weeks (45.6 (36.0) for the antrum and 63.0 (30.6) for the corpus). In contrast, IL-1β...
levels were very low in gerbils infected with the \textit{cagG} mutants throughout the observation periods (fig 7).

**DISCUSSION**

We used the Mongolian gerbil model to examine the effect of two previously unstudied genes in the \textit{cag} PAI (\textit{virD4} and \textit{cagG}) on gastric inflammation in vivo. Wild-type \textit{H pylori} caused typical severe gastritis in gerbils whereas the \textit{virD4} mutants caused very low levels of gastric inflammation, mucosal proliferative activity, and mucosal IL-1\(\beta\) levels throughout the first 12 weeks. \textit{H pylori} density was similar with the different inocula, confirming that the differences were not due to bacterial load. At 24 weeks, the degree of gastric inflammation and proliferative activity in gerbils infected with the \textit{virD4} mutants increased, reaching levels comparable with those seen at four weeks with the wild-type strains. \textit{H pylori} density in the antrum at 24 weeks in gerbils infected with the \textit{virD4} mutants was significantly higher than that with wild-type \textit{H pylori} (\(p=0.03\)). Lack of acute inflammation might help growth of the \textit{virD4} mutants; however, it remains unclear whether it is sufficient to explain the results.

Mucosal IL-1\(\beta\) levels at 24 weeks were similar for \textit{virD4} mutants and wild-type infections. IL-1\(\beta\) levels with the wild-type strains were maximal at four weeks in the corpus and at 12 weeks in the antrum. In gerbils and in mice, IL-1\(\beta\) mRNA levels do not mirror chronic mucosal inflammation. In contrast, in humans, IL-1\(\beta\) levels are consistently elevated in \textit{H pylori} infected gastric mucosa. IL-1\(\beta\) mRNA levels in the corpus were very low in the chronic phase of the infection, suggesting that induction of acute inflammation rather than inhibition of gastric acid secretion is the main role of IL-1\(\beta\) in gerbils. Probably the most important proinflammatory cytokine in the gastric mucosa is IL-8. Gerbils do not encode an IL-8 gene, as cross species RT-PCR techniques failed to identify an IL-8 gene (unpublished observation). We selected IL-1\(\beta\) based on the fact that in humans, mucosal IL-8 levels were closely correlated with mucosal IL-1\(\beta\) levels. Future studies will examine cytokine expression using IL-8 families such as KC which behave like IL-8 in mice.

With the wild-type strains, the degree of gastric inflammation reached maximal levels at 12–26 weeks and proliferative activity at four weeks. Loss of the \textit{virD4} gene temporally retarded but did not abrogate \textit{H pylori} induced gastric inflammation, and proliferative activity with the \textit{virD4} mutants was similar to that with the wild-type strains at 24 weeks. We did not examine animals beyond 24 weeks and can only speculate regarding later time points.

The \textit{virB4} (\textit{cagE}) knockout mutants produce mild gastritis and not gastric ulcers. \textit{virB4} is a major component of the type IV secretion system such that loss of the \textit{cagE} gene results in loss of CagA translocation/phosphorylation as well as loss of host cytoskeletal rearrangement and IL-8 induction. Although the \textit{virD4} mutants also lose the ability to translocate CagA into host cells, \textit{cagA} mutants can produce inflammation consistent with in vitro studies showing that CagA is not responsible for IL-8 induction. Lack of inflammation with the \textit{virD4} mutants in the first 12 weeks suggests the absence of as yet unidentified factors that translocate into epithelial cells using the type IV secretory pathway or interact with the type IV secretion system. Several factors other than the \textit{cag} PAI, in particular OipA as one of the outer membrane proteins, are related to induction of mucosal IL-8 and gastric inflammation. In addition, \textit{cag} PAI status is closely related to OipA status (for example, if the strains possess the \textit{cag} PAI, strains almost always possess functional OipA). However, possible interactions between OipA and \textit{cag} PAI were not examined in these experiments.

The \textit{cagG} mutants did not produce an inflammatory response or increase proliferative activity, most likely related to their poor ability to colonise gerbil gastric mucosa. The \textit{cagG} gene is not a \textit{vir} homologue gene and has a weak homology to the flagellar motor switch protein gene or toxin coregulated pilus biosynthesis protein gene. The current consensus is that loss of the \textit{cagG} gene also results in loss of \textit{CagA} translocation/phosphorylation. Recent reports suggest that isolates lacking \textit{cagG} genes have decreased adherence to epithelial cell lines. An in vivo study has shown no relationship between \textit{cagG} and clinical outcome; the population studied (Chinese) were predominantly infected with \textit{cag} PAI positive strains such that the effect of the \textit{cagG} gene could not be examined. Most reports, including our present study, suggest that loss of the \textit{cagG} gene results in almost complete elimination of \textit{H pylori} induced IL-8

![Hypothetical model for induction of host responses by Helicobacter pylori.](http://gut.bmj.com/)

**Figure 8**

Reduced colonisation

No functional type IV secretion system

Loss of CagA effects

No inflammation
induction. However, a recent report suggested that precise deletion of the cagA gene resulted in no reduction in IL-8 induction. Gerbils infected with cagA mutants showed no inflammation although the H pylori density score was generally equivalent to wild-type strains or virD4 mutants (fig 5). From these data it is not possible to define whether or not the lack of inflammation with cagA mutants is related to reduced colonisation, loss of the type IV secretion system, or both. Complementation experiments will be needed to resolve this issue.

Our current hypothetical model is presented in fig 8. VirD4 is thought to act as an adapter protein for the transfer of CagA protein and possibly other unknown proteins (D4 dependent) into the transfer channel formed by other Vir proteins in the cag PAI. We also hypothesise the presence of unknown proteins independent of VirD4. As virD4 mutants are unable to translocate CagA as well as any D4 dependent factors, loss of CagA effects and loss of D4 dependent factors occurs. However, it is possible that D4 independent factors may be translocated into cells or interact with the type IV secretion system, inducing chronic inflammation. cagG mutants have decreased adherence to epithelial cells and reduced ability to colonise gerbils. In addition, they are unable to translocate CagA and any D4 dependent or independent factors due to loss of functional transporter system such that inflammation would not be expected.

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Conflict of interest: None declared.

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