The Interrelationship between Cytotoxin-Associated Gene A, Vacuolating Cytotoxin, and *Helicobacter pylori*—Related Diseases

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The interrelationship between cytotoxin-associated gene A (CagA), vacuolating cytotoxin (VacA), and *Helicobacter pylori*—related diseases was investigated in 155 *H. pylori*–infected patients. Four (7%) of 60 subjects had mixed caga+ and caga- *H. pylori* infections. The *H. pylori* isolates from 98.3% of 121 patients with anti-CagA antibodies were caga+. The occurrence of caga+ *H. pylori* among 76 patients with peptic ulcer disease (PUD) was higher (93.4%) than among 79 patients with functional dyspepsia (FD; 64.6%) (odds ratio [OR] = 7.80; P < .001). VacA+ isolates were isolated from 56.6% of the PUD patients and 35.4% of the FD patients (OR = 2.37; P = .0132). For type I (caga+ VacA+) isolates, these numbers were 56.6% and 31.6%, respectively (P = .003). Only 4% of the 71 VacA- isolates were caga-. In addition, 37% of the patients with PUD were infected with caga+ VacA- *H. pylori*. χ² results did not improve when VacA was entered into the model in the presence of caga, indicating that only caga is associated with PUD.

*Helicobacter pylori*, a gram-negative, spiral-shaped, microaerophilic bacterium, infects up to 50% of the population of Western countries [1]. Although not all persons infected with the bacterium develop gastroduodenal disease, *Helicobacter* species is now recognized as the major etiologic agent of chronic active gastritis and is generally accepted as having a causative role in the pathogenesis of gastric and duodenal ulcers [2, 3, 4]. Furthermore, the bacterium is suspected of being involved in the pathogenesis of mucosa-associated lymphoid tissue [5, 6] and gastric adenocarcinoma [7].

Analysis of strains isolated from patients with peptic ulcer disease (PUD) or functional dyspepsia (FD) revealed that virtually all had genes coding for urease, flagella proteins, superoxide dismutase, mucinase, and vacuolating cytotoxin (VacA); however, in contrast to flagella proteins and urease, which are essential for colonization [8, 9], VacA is not always expressed. Expression of this cytotoxin has been linked to *H. pylori*—related PUD. Furthermore, vacuolation is induced in human epithelial cells exposed to VacA, a purified 87-kDa protein, or to strains producing this cytotoxin, and results from in vivo studies using a mouse model support the idea that VacA is involved in ulcerogenesis [10].

Another *H. pylori* protein, CagA, the product of cytotoxin-associated gene A (caga), has also been considered as a virulence factor of *H. pylori*. At the mucosal level, virtually all patients with PUD and 60% of those with FD have antibodies against this 120- to 128-kDa protein [11], which has been linked to *H. pylori*—associated gastric cancer [12, 13].

To determine the interrelationship between the *H. pylori* virulence factors CagA and VacA and the clinical outcome in *H. pylori*—related diseases, we analyzed isolates from *H. pylori*—positive subjects. Since patients can be infected with a mixed population of caga+ and caga- *H. pylori* [14], we analyzed a pool of colonies from the primary culture plate from each patient’s gastric biopsy for the presence of caga by polymerase chain reaction (PCR) and colony hybridization. The pool was also analyzed for its ability to induce vacuoles in various mammalian cell lines in culture. In addition, Western blot was used to assess the presence of anti-CagA antibodies in patient sera.

Materials and Methods

Patients. Study subjects were 155 consecutive *H. pylori*–infected adults from a group of patients who had undergone upper gastrointestinal endoscopy because of dyspeptic complaints: 76 had either an active ulcer or scars from recent ulcers, and 79 had FD.

Endoscopy, histopathology, and culture identification. During each endoscopic procedure, four antral and four corpus mucosal biopsy specimens were obtained by use of biopsy forceps, which had been sterilized (cleaned with detergent while rocking, disinfected with 70% ethanol, and autoclaved) and rinsed with sterile water after each examination. Two antrum and two corpus biopsy specimens placed in 2 mL of PBS at 4°C were used for bacteriologic assessment. Two other sets of antrum and corpus biopsies fixed in 10% formalin were examined histopathologically. Bacteriologic and histologic assessment of the mucosal biopsy specimens...
was done as previously described [14, 15]. Cultures were prepared by smearing biopsy specimens on the surface of horse blood agar plates (Colombia agar base, Oxoid CM 331; Unipath, Basingstoke, UK) and horse blood agar plates containing Skirrow supplement (Unipath). Gram-negative and oxidase-, catalase-, and urease-positive spiral or curved rods were identified as *H. pylori*.

The primary cultures of antrum and corpus biopsy specimens were separately collected using swabs. Each swab was shaken in 2 mL of 8% glycerol peptone, and the suspensions were stored at −70°C. These bacterial suspensions are referred to as frozen primary cultures [14, 16]. To minimize the effect of possible different growth rates of different *H. pylori* in the primary cultures, we used the bacterial suspensions made from the horse blood agar primary culture, which was free from contaminating microorganisms, for further analysis. For each analysis, only one additional culture from the frozen primary culture suspension was done on horse blood agar plates.

**Isolation of *H. pylori* chromosomal DNA.** After being thawed, bacterial suspensions were grown on horse blood agar plates for 3 days at 37°C in a microaerophilic environment and then harvested and resuspended in distilled water. The suspension was boiled for 5 min and centrifuged at 10,000 g for 5 min. Chromosomal DNA was isolated from the supernatant by phenol-chloroform-isomyl alcohol extraction and ethanol precipitation.

**Detection of caga by PCR.** From the published sequence for caga [17], two primers were designed complementary to sequences located within the conserved region of the gene: primer cagal, GATATGCCACCTACCCACCG (nt 1249–1270), and primer caga2, GGAAATCTTT AATCTCAGTTCGG (nt 1797–1819). The primers were used in a standard PCR mixture and produced a calculated product of 570 bp. In brief, 10 ng of DNA was used in a 25-µL PCR mixture containing 10 mM TRIS-HCl (pH 8.8), 50 mM KCl, 3.0 mM MgCl2, and 0.1 mg/mL bovine serum albumin. The reaction conditions were as follows: 40 cycles of 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C. The PCR products were analyzed by horizontal agarose (2%) gel electrophoresis as previously described [16].

**Detection of caga by colony hybridization.** An aliquot from the bacterial suspension prepared from the primary culture plate of the biopsy specimens from each patient was grown on horse blood agar plates for 3 days. Approximately 200–400 colonies were transferred from the plates onto a nylon membrane (Zeta probe; Bio-Rad, Upplands Vasby, Sweden) by replica plating. The colonies were lysed and denatured essentially as described by Sambrook et al. [18].

In brief, filters were placed on 3MM paper (Whatman, Maidstone, UK) saturated with 10% SDS for 5 min, transferred to a second sheet of paper saturated with 0.5 M NaOH and 1.5 M NaCl, and incubated for 10 min. The filters were neutralized by incubating on paper saturated with 1.5 M NaCl and 0.5 M TRIS, pH 8.0, for 5 min. The filters were then transferred to paper saturated with 2X SSPE (300 mM NaCl, 20 mM NaH2PO4 • 2H2O, and 2 mM EDTA [pH 7.4]) and incubated for 5 min. The filters were allowed to dry at room temperature and then were baked at 120°C for 1 h.

The *caga* detection probe was made by PCR using cagal and caga2 as primers. The PCR products obtained from a number of strains were pooled and run on a 1% agarose gel. After electrophoresis, the 570-bp PCR product was excised from the gel and extracted from the agarose using Qiaex (Qiagen, Chasworth, CA) according to manufacturer’s instructions. The probe was labeled with digoxigenin (Boehringer, Mannheim, Germany) at its 3’ ends, according to manufacturer’s instructions. After being hybridized at 68°C for 18 h in 5× SSC (20× SSC is 3 M NaCl and 0.3 M sodium citrate, pH 7.0) with 1% blocking reagents (Boehringer), 0.02% SDS, and 0.1% N-lauroylsarcosine, the blots were washed 2 times with 2× SSC with 0.1% SDS at 50°C. Probes were detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase and stains according to manufacturer’s instructions.

**Detection of anti-CagA antibodies in sera by Western blot analysis.** Lysates of caga+ and caga− *H. pylori* strains, characterized previously [14], were made by heating the cells resuspended in electrophoresis sample buffer (0.25 M TRIS, pH 6.8, 2% SDS, 10% glycerol, and 0.2% bromophenol blue) at 100°C for 5 min. The bacterial proteins were separated on a 7.5% SDS polyacrylamide gel and transferred to nitrocellulose ( pore size, 0.45 µm; Schleicher & Schuell, Dassel, Germany) in 10 mM NaHCO3, 3 mM Na2CO3, and 20% methanol at 50 mA for 18 h. The blots were blocked with blot buffer (10 mM TRIS-HCl, pH 7.5, 0.5 M NaCl, and 0.5% Tween 20) and incubated with patient serum (1:1000 in blot buffer). After being washed with blot buffer, the blots were incubated with α-human IgG–alkaline phosphatase conjugate (1:7500 dilution in blot buffer; Promega, Madison, Wisconsin). Positive bands were visualized by incubation with 165 µg/mL 5-bromo-4-chloro-3-indolyl-phosphate, p-toluidine salt (Promega), and 330 µg/mL nitroblue tetrazolium chloride (Promega) in 0.1 M TRIS-HCl, pH 9.5, 0.1 M NaCl, and 5 mM MgCl2.

**Vacuolating activity assay.** HMO2 (human gastric) [19], Chang (human conjunctivae), HeLa (human endocervix), and H-292 (human bronchus epithelium) cells were cultured in separate 24-well plates to 80% confluency in L-15 medium, Dulbecco’s modified Eagle medium (DMEM), and RPMI, respectively. Each cell culture medium was supplemented with 5% fetal calf serum (FCS). Media were replaced by DMEM and 5% FCS (1 mL) 16 h before exposure of the cells to 1.107–2.107 cfu of bacteria, as determined by optical density. Under these conditions, the density of *H. pylori* increased from 107 to 109 cfu/mL within 8 h and to 1010 cfu/mL within 24 h. The results of this assay were compared with those of Leunk et al. [20], who used a 1:10 dilution of a 30-fold concentrated supernatant of a broth culture of *H. pylori* grown for 48 h: Results were identical for 10 VacA+ and VacA− *H. pylori*. Vacuolization was assessed by light microscopy 24 and 48 h after exposure of the eukaryotic cells to the bacteria. *H. pylori* isolates inducing vacuoles in >50% of the cells were considered to be positive for vacuolating activity.

**Statistical methods.** Proportions were compared by χ2 analysis with Yates’s correction. The correlation of caga and VacA with PUD was further analyzed by use of stepwise logistic regression, using PUD as the dependent variable [21].

**Results**

**Prevalence of caga+ *H. pylori* isolates in patients with PUD or FD.** The presence of caga in the *H. pylori* isolates from 155 patients, of whom 76 had an endoscopically proven PUD, was determined by PCR. Patients can carry mixed populations of caga+ and caga− *H. pylori* [14]; therefore, to determine the
Anti-CagA antibodies were detected in sera from all cagA+ H. pylori–infected patients (n = 119) and from only 2 (6%; both with FD) of the 32 cagA+ H. pylori–infected patients. Sera from 20 patients from whom cagA+ H. pylori were isolated were tested with a CagA-positive control strain and with their own cagA+ H. pylori isolate in immunoblotting. Antibodies in each serum sample recognized the CagA protein of both strains. The molecular mass of the CagA proteins of these 20 strains varied (110–140 kDa). This diversity is probably due to the reported variation in the numbers of internal repeat units in the gene coding for this protein [13, 17].

Overall, the sera from 93.2% (68/73) of the patients with PUD and from 67.9% (53/78) of the patients with FD contained anti-CagA antibodies.

Vacuolating capacity of H. pylori isolated from patients with PUD or FD. The H. pylori isolates from all patients were tested for their vacuolating capacity on 4 different epithelial cell lines. Of the 155 isolates, 71 (45.8%) induced vacuoles in Chang, HeLa, and H-292 cells and were therefore considered to be VacA+. However, in the gastric epithelial HM02 cells, these 71 H. pylori isolates caused contraction of the cells rather than vacuoles in the cytoplasm of the cells. Analysis showed that the VacA+ H. pylori occurred more frequently among patients with PUD (56.5%, 43/76) than among those with FD (35.4%, 28/79) (odds ratio = 2.37; P = .0132). The prevalences of Vac+ H. pylori among patients with UD (62%, 34/55), UV (43%, 6/14), or both (43%, 3/7) were similar.

Interrelationship between cagA-, Vac-, and H. pylori–related diseases. Data summarized in table 1 show that 95.8% (68/71) of the cytotoxins-producing H. pylori isolates are cagA+.

Stepwise logistic regression analysis of the data showed that when cagA was present and VacA was entered into the model, \( \chi^2 \) results did not significantly improve (1.12; P = .290), indicating that only cagA or a factor(s) related to this gene or its product are associated with PUD.

Discussion

To our knowledge, this study is the first to describe the interrelationship between cagA, CagA, and VacA of H. pylori and PUD in a large cohort of patients. H. pylori isolates from 76 patients with PUD and 79 with FD were analyzed by PCR and colony hybridization techniques for the occurrence of cagA, by Western blot for the expression of CagA by detecting antibodies to CagA in patient sera, and by a vacuolization assay for the expression of VacA in various epithelial cell lines. The results show that almost all patients with PUD (93.4%) were infected with cagA+ H. pylori, that almost all VacA+ H. pylori isolates (95.8%) were cagA+ and that cagA+ H. pylori were
isolated from 98.3% (119/121) of the patients with anti-CagA antibodies (table 1). Last, the occurrence of cagA+ H. pylori is significantly higher among patients with PUD than among patients with FD.

These data support the conclusion that PUD can be linked to CagA protein expression and are consistent with the results of Crabtree et al. [11], who initially found that almost all patients with PUD had anti-CagA antibodies in their gastric mucosa. In contrast to our findings, it was recently reported that cagA+ H. pylori could be isolated from patients with PUD and from patients with FD with similar frequencies [22]. In addition, others reported that only cagA− H. pylori could be isolated from a substantial number of patients with anti-CagA antibodies in their sera [23, 24]. It has been shown that patients can be infected with a mixed population of cagA+ and cagA− H. pylori [14]; therefore, in this study, a pool of colonies from the primary culture plate were analyzed to assure that cagA+ H. pylori among a mixed population were detected. Mixed infection was observed in 7% (4/60) of the H. pylori populations tested. Furthermore, the results show a nearly perfect match between anti-CagA antibodies in the sera of the patients and the presence of cagA in their H. pylori isolate, and they show a strong relationship between cagA+ H. pylori and CagA and the presence of PUD.

The 5 patients with PUD without anti-CagA antibodies in their serum specimens were infected with cagA− H. pylori. Of the 79 patients with FD, 64.6% were infected with cagA+ H. pylori. These findings illustrate that although being strongly associated with PUD, cagA and CagA are not essential for PUD and probably, apart from cagA and its gene product, other host factors are also important to the development of PUD.

The serum of all patients infected with cagA+ H. pylori contained anti-CagA antibodies. Two patients with FD had anti-CagA antibodies, although they were infected with cagA− H. pylori. Most likely, these patients were infected by a mixed population of cagA+ and cagA− H. pylori, of which the proportion of cagA+ H. pylori was too small to be detected by our assay. It is also possible that the cagA+ H. pylori in these patients was lost with time.

In this study, 46% of the H. pylori isolates induced vacuoles in epithelial cells of nongastric origin, as most notably seen in HeLa cells. In contrast, the same isolates did not induce vacuoles in gastric epithelial HM02 cells; however, they did cause contraction of the cells. A similar finding has been reported for other gastric epithelial cell lines (KATO-3, ST-42, AGS) exposed to H. pylori strains that induced vacuoles in HeLa cells [25]. The mechanism of this difference in response of gastric versus nongastric epithelial cells exposed to VacA+ H. pylori remains to be elucidated. It would be interesting to know whether bafilomycin A1, a compound inhibiting the vacuolar ATPase proton pump and, thus, the induction of vacuoles in HeLa cells by cytotoxic H. pylori [26], can also prevent contraction of gastric epithelial cells, indicating a similar mechanism involved in vacuolization and contraction. Another explanation may be that a factor coexpressed with VacA is responsible for the difference in cytotoxic effect on gastric epithelial cells compared with other epithelial cell lines [27].

In this study, 46% of all H. pylori populations produced VacA. Furthermore, 57% of the PUD patients and 35% of the FD patients were infected with Vac+. H. pylori. These results are in agreement with previously reported proportions (range, 28%–55%) of Vac+ H. pylori among tested isolates [20, 23, 28–30]. In addition, the proportion of Vac+ H. pylori was 46%–67% and 13%–46% among patients with PUD and FD, respectively [28–30].

Of 71 cytotoxic H. pylori isolates, 68 (95.8%) were cagA+. The finding that 3 cagA− H. pylori isolates were VacA− is consistent with the notion that CagA does not affect the VacA activity of H. pylori [31]. In contrast, 44% (54/122) of the cagA+ H. pylori isolates were cytotoxix negative; 50% of these were isolated from patients with PUD. The number of cagA+VacA− H. pylori populations we found was slightly higher than that found by Xiang et al. [23] and Owen et al. [22], who showed that 24% (8/33) and 39% (15/38), respectively, of their cagA+ strains were VacA-. This difference might be due to the use of a less sensitive vacuolating assay. In this study, H. pylori (107 cfu/mL) were directly added to eukaryotic cells rather than to a 1:10 dilution of a 30-fold concentrated culture supernatant [20, 27]. In the presence of cells, H. pylori rapidly grows to high densities (109 and 1011 cfu/mL within 8 and 24 h, respectively).

In addition, as previously mentioned, the proportion of Vac+ H. pylori among all patients and among patients with PUD or FD is consistent with previously reported data.

It has been proposed that clinical H. pylori isolates be divided into 2 major types of bacteria [23, 32], type I, containing and

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NOTE. ND, not done.
* Expresses only cagA or VacA [23].

Table 1. Characterization of H. pylori isolates and anti-CagA antibody in sera from patients with defined gastroduodenal pathology and distribution of characterized H. pylori according to disease entity, peptic ulcer disease (PUD) or functional dyspepsia (FD), at endoscopy.
expressing cagA and expressing vacA, and type II, not containing cagA and not expressing vacA, and an intermediate type, expressing only CagA or VacA. The results of this study showed that patients with PUD were mainly infected with type I (56.6%, 43/76) and intermediate type (36.8%, 28/76) bacteria. Type II was present in only 6.6% (5/76) (table 1). Among patients with FD, the type I and II and intermediate phenotypes of H. pylori were evenly distributed: 31.6% (25/79), 29.1% (23/79), and 39.2% (31/79), respectively. These results suggest an association between type I H. pylori and PUD. The presence of VacA* was strongly associated with cagA*: almost all VacA H. pylori isolates were cagA*. However, stepwise logistic regression analysis of the data indicated that only cagA or a factor(s) related to this gene or its product is associated with PUD.

References