

## Co-expression in *Helicobacter pylori* of *cagA* and non-opsonic neutrophil activation enhances the association with peptic ulcer disease

D Danielsson, S M Farmery, B Blomberg, S Perry, H Rautelin, J E Crabtree

### Abstract

**Aims**—To investigate the association of *cagA* positivity and non-opsonic neutrophil activation capacity in wild-type *Helicobacter pylori* strains with peptic ulcer disease or chronic gastritis only.

**Methods**—*Helicobacter pylori* were isolated from antral biopsies of 53 consecutive patients with chronic antral gastritis, of whom 24 had peptic ulcer disease endoscopically. The presence of *cagA*, a marker for the *cag* pathogenicity island, was determined by polymerase chain reaction with specific oligonucleotide primers, and non-opsonic neutrophil activation capacity by luminol enhanced chemiluminescence.

**Results**—The *cagA* gene was present in 39 of 53 (73.6%) strains, 20 of which (83.3%) were from the 24 patients with peptic ulcer disease and 19 (65.5%) from the 29 patients with chronic gastritis only. Non-opsonic neutrophil activation was found in 29 (54.7%) strains, 16 of which (66.7%) were from patients with peptic ulcer disease, and 13 (44.8%) from those with chronic gastritis. Non-opsonic neutrophil activation was found more frequently in *cagA*<sup>+</sup> than *cagA*<sup>-</sup> strains (59% *v* 42.9%). Whereas four of the 14 *cagA*<sup>-</sup> strains and eight of the 24 non-opsonic neutrophil activation negative strains were from patients with peptic ulcer disease, only two of 24 (8.3%) peptic ulcer disease strains expressed neither *cagA* nor non-opsonic neutrophil activation. The *cagA* gene and non-opsonic neutrophil activation capacity were co-expressed in 14 of 24 (58.3%) strains from patients with peptic ulcer disease, and in nine of 29 (31%) strains from individuals with chronic gastritis.

**Conclusions**—Positivity for *cagA* and non-opsonic neutrophil activation occur independently in wild-type *H pylori* strains. However, co-expression of the two markers enhanced the prediction of peptic ulcer disease.

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Keywords: *Helicobacter pylori*; neutrophil; *cagA*

There is convincing evidence that *Helicobacter pylori* infection is the main cause of peptic ulcer disease and a very important cofactor for the development of gastric cancer.<sup>1-3</sup> It is also one of the world's most common bacterial infec-

tions. However, only a small number of infected individuals will suffer from clinically overt gastroduodenal disease and the reasons for this are unclear. The production of large amounts of urease and the presence of flagella (four or five) for swimming through the mucous layer<sup>4,5</sup> are seen in all clinical isolates, and are necessary for the colonisation of the stomach and survival in this hostile environment. In addition to these putative factors, which might explain why all infected persons develop chronic superficial gastritis with accumulation of lymphocytes, macrophages, and plasma cells, there is increasing evidence that some strains are more virulent than others.

The extent of the chronic gastritis is determined by the infiltration of neutrophils in the gastric mucosa and epithelial layer, which might be important factors for tissue damage and ulceration.<sup>6</sup> *CagA* is a phenotypic marker for proinflammatory *H pylori* strains that induce production of interleukin 8 (IL-8) in gastric epithelial cells.<sup>7,8</sup> Secreted IL-8 will bind to proteoglycans within the matrix of lamina propria and, together with *H pylori*, will attract neutrophils and facilitate their migration towards the epithelium.<sup>9</sup> In the search for virulence factors to predict clinical outcome, many studies have demonstrated the presence of *cagA*, a marker for the *cag* pathogenicity island,<sup>10,11</sup> and particular alleles of the *vacA* gene to be associated with peptic ulcer disease and gastric cancer,<sup>12-15</sup> but other studies have produced conflicting results.<sup>16</sup>

Particular strains of *H pylori* have non-opsonic neutrophil activating capacity, and it was shown that such strains are more often isolated from individuals with peptic ulcer disease than from those with chronic gastritis only.<sup>17</sup> Our earlier studies showed this association was independent of the production of vacuolating cytotoxin.<sup>18</sup> In our present study, we have examined the incidence and co-expression of *cagA* and non-opsonic neutrophil activation in wild-type *H pylori* strains isolated from patients with peptic ulcer disease and chronic gastritis only.

### Materials and methods

#### HELICOBACTER PYLORI STRAINS AND PATIENTS

A total of 53 wild-type *H pylori* strains were included in our study. They were all isolated from antral biopsies taken from 53 individual patients referred for upper gastrointestinal endoscopy at the division of gastroenterology, department of internal medicine, Örebro Medical Centre Hospital, Örebro, Sweden.

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Table 1 Oligonucleotide primers for *cagA* and *ureA* PCR

Gene	Accession number	Primer	Position	Expected product size
<i>cagA</i> Sense	X 70039	GATAACGCTGTCGCTTCATACG	631–652	409
		CTGCAAAGATTGTTTGGCAGA	1039–1018	
<i>ureA</i> Sense	M 60398	GCCAATGGTAAATTAGTT	2962–2979	411
		CTCCTTAATTGTTTTTAC	3372–3355	

Forty nine of the strains had been used in a previous study.<sup>17</sup> None of the patients had previously received eradication treatment for *H pylori* infection, and patients on non-steroidal anti-inflammatory drugs (NSAIDs) were excluded. Histologically, all patients had chronic antral gastritis. Endoscopically, 24 patients had peptic ulcer disease; 10 patients had a duodenal ulcer, one of whom also had a gastric ulcer; seven patients had a prepyloric ulcer; five patients had a gastric ulcer; two patients had a pyloric ulcer, one of whom also had a gastric ulcer.

All the strains showed typical colony morphology, were oxidase, catalase and urease positive, and identified as typical curved rods by Gram stain. For the in vitro experiments, the strains were grown on brain heart infusion agar with 10% horse blood, without antibiotics, in a microaerophilic atmosphere for two or three days at 37°C. The individual strains were stored in preservation medium at –70°C, and subculturing was kept at a minimum.

#### NEUTROPHILS

Heparinised blood from healthy blood donors was used to prepare neutrophils by Ficoll-Hypaque (Pharmacia and Upjohn, Uppsala, Sweden) centrifugation in accordance with the method of Böyum,<sup>19</sup> slightly modified as described previously.<sup>20</sup> For each series of experiments, neutrophils were prepared and pooled from three blood donors of the same blood group (A Rh<sup>+</sup> or O Rh<sup>+</sup>). Neutrophils were suspended in 0.01 M phosphate buffered 0.15 M saline (PBS) supplemented with MgCl<sub>2</sub>, CaCl<sub>2</sub>, glucose, and gelatin (PBS-GG) as described previously.<sup>20</sup> The purity and viability of the neutrophils exceeded 95%.

#### CHEMILUMINESCENCE

Luminol enhanced chemiluminescence was used as described previously to measure the oxidative burst of neutrophils induced by non-opsonised *H pylori* organisms.<sup>17</sup> Briefly, 300 µl of PBS-GG, 100 µl of neutrophils (5 × 10<sup>6</sup>/ml), 50 µl of non-opsonised *H pylori* organisms (5 × 10<sup>8</sup>/ml), and 50 µl of 10<sup>-5</sup> M luminol (Sigma, St Louis, Missouri, USA) were added to each test tube (LKB, Bromma, Sweden). The measurements with a luminometer (LKB Wallac 1251; LKB, Turku, Finland) were always started within one minute after the bacterial suspension had been added. The assays were performed at 37°C, and chemiluminescence from each sample was measured at 60–90 second intervals during a period of 30 minutes. The *H pylori* strains 11637 and C-7050 were used as positive and negative controls, respectively, in each run.<sup>17</sup> A maxi-

mum peak response of at least 20% or more of the reference strain 11637 was regarded as positive.

#### DNA PREPARATION AND POLYMERASE CHAIN REACTION (PCR) DETECTION OF *cagA*

Bacterial colonies from each subject were harvested into PBS, pelleted by centrifugation, and stored at –70°C until assayed. DNA was extracted from the bacterial pellets using phenol/chloroform and precipitated. The presence of *cagA* was determined by PCR using *cagA* specific primers (table 1), as described previously.<sup>21</sup> PCR amplification of *ureA* was used as a positive control. In each PCR assay, DNA extracted from the *cagA* positive strain NCTC 11637 and the *cagA* negative G50 strain<sup>7</sup> was used as positive and negative controls, respectively. Strains were considered *cagA* positive when the product of expected size was observed.

#### STATISTICS

Fischer's exact test was used for the statistical calculations.

#### Results

Tables 2 and 3 show the presence of the *cagA* gene and non-opsonic neutrophil activation in the 53 *H pylori* strains, individually and combined, in the 24 patients with peptic ulcer disease and the 29 non-ulcer patients with chronic gastritis only.

The *cagA* gene was present in 39 of 53 (73.6%) strains, 20 of which (83.3%) were from patients with peptic ulcer disease, and 19 (65.5%) from patients with chronic gastritis only. The corresponding figures for *cagA* negative strains were four (16.7%) and 10 (34.5%), respectively (table 2). The difference between the patient groups was not significant ( $p = 0.212$ ).

Non-opsonic neutrophil activation capacity was found in 29 of 53 (54.7%) strains, 16 of which (66.7%) were from patients with peptic ulcer disease, and 13 (44.8%) from patients with chronic gastritis alone. The corresponding

Table 2 Expression of *cagA* gene and non-opsonic neutrophil activation capacity (NAC) in *Helicobacter pylori* strains from patients with peptic ulcer disease (PUD) or chronic gastritis (CG)

<i>H pylori</i> markers	Patients			
	PUD	CG	Total	
<i>cagA</i> gene	+	20	19	39
	–	4	10	14
NAC	+	16	13	29
	–	8	16	24

Table 3 Expression of *cagA* gene and non-opsonic neutrophil activation capacity (NAC), individually and combined, in 53 wild-type *Helicobacter pylori* strains from patients with peptic ulcer disease (PUD) or chronic gastritis (CG)

	Expression				Total
	+	–	+	–	
<i>cagA</i> gene	+	–	–	–	
NAC	+	–	+	–	
PUD	14	6	2	2	24
CG	9	10	4	6	29
Total	23	16	6	8	53

figures for strains without non-opsonic neutrophil activation capacity were eight (33.3%) and 16 (55.2%), respectively (table 2). The difference between the patient groups was not significant ( $p = 0.166$ ).

Non-opsonic neutrophil activation was found more frequently in *cagA*<sup>+</sup> than *cagA*<sup>-</sup> strains (59% *v* 42.9%). Whereas four of the 14 *cagA*<sup>-</sup> strains and eight of the 24 non-opsonic neutrophil activation negative strains were from patients with peptic ulcer disease, only two of 24 (8.3%) peptic ulcer disease strains expressed neither *cagA* nor non-opsonic neutrophil activation (tables 2 and 3). The *cagA* gene and non-opsonic neutrophil activation were co-expressed in 14 of 24 (58.3%) strains from patients with peptic ulcer disease, and in nine of 29 (31%) strains from individuals with chronic gastritis. Only one marker—that is, either *cagA* or non-opsonic neutrophil activation capacity—or none, was expressed in 10 (41.7%) strains from patients with peptic ulcer disease, and in 20 of 29 (69%) strains from individuals with chronic gastritis only. This gives a *p* value of 0.056 between the two patient groups (Fischer's exact test).

### Discussion

Neutrophils are important cells in the first line of defence against invading microorganisms in general, and pathogenic bacteria in particular. When stimulated to phagocytose they produce an array of reactive oxygen metabolites and release highly biologically active enzymes and other factors, which can be detrimental not only to invading microbes but also to cells and tissues of the host.<sup>22</sup> *Helicobacter pylori* strains with the *cag* pathogenicity island are associated with an enhanced neutrophil response *in vivo*,<sup>12,23</sup> probably because of their ability to induce IL-8 production in gastric epithelial cells.<sup>7-11</sup> Therefore, we hypothesised that the co-expression of *cagA* and non-opsonic neutrophil activation capacity in wild-type clinical *H. pylori* isolates might be related to the mucosal damage associated with *H. pylori* infection. Our findings support this view, and co-expression of *cagA* and non-opsonic neutrophil activation enhanced the prediction of peptic ulcer disease as compared with chronic gastritis only.

The mosaicism of the *cagA* genotype and non-opsonic neutrophil activation capacity among the clinical isolates was obvious. One or both of these factors occurred in 45 of 53 (84.9%) strains, and they were more frequently found in patients with peptic ulcer disease than in individuals with chronic gastritis only (91.7% *v* 79.3%). The lack of a significant association between non-opsonic neutrophil activation and peptic ulcer disease is in contrast to one previous study.<sup>17</sup> However, only 49 of the 55 strains used in our previous study were included in our present study. Nonetheless, non-opsonic neutrophil activation was more frequent in patients with peptic ulcer disease than in individuals with chronic gastritis (66.7% *v* 44.8%).

*Helicobacter pylori* are broadly divided into type I and type II strains, the former character-

ised by the presence of *cagA*, a marker of the *cag* pathogenicity island,<sup>10,11</sup> and the production of vacuolating cytotoxin, *VacA*.<sup>24</sup> In between the two extremes of type I and type II, there are a number of subpopulations of intermediate strains.<sup>24</sup> In studies to predict the clinical outcome of *H. pylori* infection, considerable attention has been paid to *vacA* and *cagA* genotypes.<sup>15,16,21,25</sup> Even though many studies from Europe and the USA show particular *vacA* genotypes to be significantly more frequent in patients with peptic ulcer disease than in individuals with non-ulcer dyspepsia,<sup>15,26</sup> there are studies, particularly from countries in Asia, with conflicting results.<sup>27,28</sup> In earlier studies, we found that all the strains with vacuolating cytotoxin activity were positive either for non-opsonic neutrophil activation or for *cagA*, or both (D Danielsson *et al.* Presented at the North American Helicobacter pylori meeting, Foundation for Gastrointestinal Microbial Pathogens; Phoenix Arizona, February 27–28, 1998).<sup>18</sup> In fact, more than 80% of cytotoxin producing strains co-expressed the *cagA* gene and non-opsonic neutrophil activation. Even though *CagA*, non-opsonic neutrophil activation, and the *VacA* cytotoxin may occur independently, it is obvious that the concomitant occurrence of two or all three markers will enhance the prediction of clinical outcome. Whereas the genetic loci for *cagA* and *vacA* are known, the gene encoding non-opsonic neutrophil activation capacity remains to be determined.

In summary, co-expression in *H. pylori* strains of non-opsonic neutrophil activation and the *cag* pathogenicity island will enhance gastric mucosal inflammation, and together with the vacuolating cytotoxin could be considered as virulence factors that can be used to predict clinical outcome.

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