Association of cytotoxin production and neutrophil activation by strains of *Helicobacter pylori* isolated from patients with peptic ulceration and chronic gastritis

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Abstract

Background—*Helicobacter pylori* is associated with neutrophil infiltration within the gastroduodenal mucosa. Neutrophil activation provides a major source of oxygen free radicals, which have been implicated in the pathogenesis of peptic ulceration.

Aim—To investigate if cytotoxin producing strains of *H pylori* are associated with the generation of oxidative burst in polymorphonuclear neutrophils (PMNs).

Patients—76 patients undergoing endoscopy of whom 45 had peptic ulcer and 31 chronic gastritis only were studied.

Methods—Strains of *H pylori* were cultured in Brucella broth. After 48 hours, bacteria were harvested by centrifugation and a bacterial suspension prepared as a stimulus for PMN oxidative burst using chemiluminescence. PMNs were prepared from healthy blood donors. To test the ability of strains to produce cytotoxin, culture supernatants of each were concentrated by polyethylene glycol and tested on cultured Vero cells for intracellular vacuolation.

Results—30 of 45 (66.7%) peptic ulcer patients induced cell vacuolation versus nine of 31 (29%) strains from patients with chronic gastritis only ($p < 0.01$). Cytotoxin positive strains of *H pylori* regardless of the presence or absence of peptic ulcer displayed an increased induction of respiratory burst in PMNs compared with toxin negative strains from patients with chronic gastritis only ($p < 0.05$). Among the toxin negative strains, those from patients with peptic ulcer did not show a significant increase of the oxidative burst than those from patients without peptic ulcer (NS).

Conclusion—Toxinogenicity of strains of *H pylori* seems to be correlated with neutrophil respiratory burst and peptic ulceration. The ability of some strains of *H pylori* to produce cytotoxin and to induce the oxidative burst in neutrophils may be important in the pathogenesis of peptic ulcer disease.

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Keywords: *Helicobacter pylori*, cytotoxin, free oxygen radicals, peptic ulcer.

The mechanisms by which *Helicobacter pylori*

causes gastroduodenal mucosal damage are not well established. Free oxygen radicals have been implicated in a wide spectrum of human disease,¹ including gastrointestinal disorders^{2 3} and may play an important part in gastroduodenal inflammation and peptic ulcer.⁴ A prominent histological feature of *H pylori* infection is a dense infiltration of polymorphonuclear cells in the epithelium and the underlying lamina propria, which could be an important source of free radicals.^{5 6} Once activated, the neutrophils release free oxygen radicals, which may damage mucosal integrity.⁷ There is evidence to show that *H pylori* stimulates gastric antral mucosal reactive oxygen radical production in vivo.⁸

Several potential bacterial virulence factors of *H pylori* may contribute to gastroduodenal mucosal damage, such as cytotoxin and urease.^{9 10} Recent studies suggest that cytotoxin is associated with peptic ulceration and chronic active gastric inflammation, as is the CagA protein, which is strongly associated with cytotoxicity.^{11 12}

A previous report¹³ showed that *H pylori* surface proteins that contain urease could activate blood monocytes, leading to the secretion of inflammatory cytokines and reactive oxygen intermediates, although the protein(s) that serve as the monocyte activator is not known. Another study shows that there is a protein with an estimated molecular weight of 150 kDa, which is associated with neutrophil activation.¹⁴

In this study, we have investigated if a relation exists between cytotoxin producing strains of *H pylori* and the generation of free oxygen radicals by neutrophils and their relation to peptic ulcer disease.

Methods

Patients

Seventy six patients undergoing endoscopy in Glasgow Royal Infirmary were recruited in this study. These consisted of 45 with peptic ulcer (36 duodenal ulcer, nine gastric ulcer), and 31 with chronic gastritis only. Forty four patients were male (age between 20-75, mean 57), and 32 were female (age 24-73, mean 55). At endoscopy, gastric biopsy specimens were taken for culture, CLO test (Delta West Ltd, Australia), and histological examination. This study was approved by the Glasgow Royal Infirmary University of Glasgow NHS Trust Ethics Committee.

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Isolation of *H. pylori*

Seventy six clinical isolates of *H. pylori* were studied. All the strains were positive for urease, oxidase, catalase, and showed typical morphology under light microscopy. For cytotoxin measurement and chemiluminescence assay, the strains were cultured in Brucella broth (Unipath, Basingstoke) containing 5% fetal bovine serum (Sigma, UK) at 37°C under microaerophilic conditions using a BBL Campypak (Cokeyville, USA), on a rotary shaker (150 rpm). After 48 hours, the broth cultures were examined under microscopy for the typical morphology of curved bacilli and the percentage of coccoid formed strains in each culture was recorded. The percentage was found to be similar for all the strains in this study, which was 18–20%. The cultures were then centrifuged at 3000 rpm for 15 minutes, bacteria collected for chemiluminescence assay, and culture supernatants for cytotoxin assay. A broth culture without the inoculation of *H. pylori* was also prepared in the same way.

Cytotoxin assay

Culture supernatants of *H. pylori* were collected and filtered through 0.4 µm filters (Millipore, France). The culture supernatants were then transferred to dialysis tubing (Sigma, UK) capable of retaining molecules with molecular weight greater than 12 kDa and concentrated 20-fold by reverse dialysis versus polyethylene glycol (Sigma, UK) as described earlier.¹⁵ Cultured Vero cells in Earle's medium (Gibco, Paisley) were used to measure intracellular vacuolation attributable to the cytotoxin according to the methods of Cover *et al.*¹⁶ Each concentrated culture supernatant was diluted from 1:5 to 1:160 and incubated for 24 hours with Vero cells in 96 well tissue culture plates. Cell vacuolation was quantified both visually and spectrophotometrically by following the uptake of neutral red. The maximum dilution of supernatant that produced vacuolation was defined as the titre of cytotoxin. During the assay, the broth culture supernatant without the inoculation of *H. pylori* was used as a toxin negative control.

Chemiluminescence assay

Fresh cultures of *H. pylori* were washed three times with saline. A bacterial suspension was prepared and adjusted to a concentration of 5×10^7 /ml. Healthy young adults without a history of peptic ulcer and under no treatment were used as white cell donors. Venous blood was separated by density gradient centrifugation (Polymorphprep, Nycomed, Birmingham, UK) and the polymorphonuclear leucocytes were washed twice in gel-Hanks's solution and standardised to contain 1×10^7 /ml. Free oxygen radical formation by polymorphonuclear neutrophils (PMNs) in response to *H. pylori* was studied by luminol dependent chemiluminescence assay. Seventy six non-opsonised strains and 42 strains opsonised with 10% normal human serum were tested in parallel in the test. The oxidative burst of

PMNs was recorded in cps (count per second) in a Luminometer (Canberra Packard, Caversham, UK). The peak value (cps) and the time (min) to reach the peak value were recorded for each strain. The assay for each sample was counted at three to four minute intervals for at least 60 minutes and all the bacterial suspensions were tested three times.

Statistical methods

The χ^2 test and Student's *t* test were used for two group comparisons. A two way analysis of variance and multiple comparison procedure was used to determine the independent effect of toxinogenicity and the presence or absence of peptic ulcer on the chemiluminescence response induced by strains of *H. pylori*.

Results

Production of cytotoxin

Concentrated culture supernatants from 39 of 76 (51.3%) of the isolates of *H. pylori* produced measurable cell vacuolation. Among the strains from peptic ulcer patients 30 of 45 (66.7%) induced cell vacuolation versus nine of 31 (29%) strains from patients with chronic gastritis only ($p < 0.01$) (Table I). Some variation in toxinogenicity of the various strains was seen with titres of toxin ranging from 0 to 160. The mean titre of cytotoxin produced by the strains from patients with peptic ulcer was 22.67 versus 7.74 in patients with chronic gastritis only ($p < 0.01$, 95% CI 4.96, 24.9).

Generation of reactive oxygen radicals

The Figure shows the chemiluminescence response induced by 76 non-opsonised strains of *H. pylori*. It shows that there is strain to strain variation in the ability to induce the oxidative burst in PMNs displayed by the difference in the peak count (kcps) and the time to reach peak (min). Some produced a stronger and more rapid response than others. The chemiluminescence pattern for each strain was reproducible. Although there are some small variations in peak height for each strain on each test occasion, the peak time was fairly constant (Table II).

Reactive oxygen radicals, cytotoxin production, and the presence of peptic ulcer disease

Using a two way analysis of variance approach, the effect of toxin status and the presence or absence of peptic ulcer on each strain's ability

TABLE I Cytotoxin production by strains of *H. pylori* isolated from 76 patients with either peptic ulcer or chronic gastritis only

Toxinogenicity of strains	Prevalence among patients with	
	Peptic ulcer	Chronic gastritis only
Tox+	30/45*	9/31
Tox-	15/45	22/31

* $p < 0.01$, compared with patients with chronic gastritis only (χ^2 test).

Neutrophil respiratory burst (as peak count of chemiluminescence) with 76 clinical isolates of *H. pylori*. Results are the mean of three separate experiments and shown as (kilocount per second, kcps) and the time to reach peak (min).

to induce a chemiluminescence response in terms of peak count and the time to reach peak were investigated. Table III summarises and shows that the chemiluminescence response in terms of peak count induced by toxin positive strains regardless of the presence or absence of peptic ulcer (groups 1 and 2) is higher than that induced by those toxin negative strains from patients without peptic ulcer (group 4) ($p < 0.05$). It also shows that toxin positive strains (groups 1 and 2) take less time to induce peak chemiluminescence than those strains of group 4 ($p < 0.05$). These results show that the toxinogenicity of *H. pylori* may have a significant effect on the chemiluminescence response, although there is no significant difference between toxin positive strains (groups 1 and 2) and group 3, which were toxin negative but associated with peptic ulceration ($p > 0.05$). However, among the

toxin negative strains, those from patients with peptic ulcer failed to show any significant enhancement of the chemiluminescence response compared with those from patients without peptic ulcer (group 4) ($p > 0.05$) in terms of peak count and the time to reach peak.

When opsonised with 10% human serum, most strains of *H. pylori* resulted in a stronger and more rapid chemiluminescence response than non-opsonised, whether or not the test serum contained any *H. pylori* antibody. A few of (seven of 42) strains that induced a high chemiluminescence response when non-opsonised gave a slightly decreased peak count (mean (SD) decrease: 65.4 (36.5)), while the time to reach peak was relatively unchanged. As can be seen from Table IV, there is no significant difference between the four groups of strains ($p > 0.05$), indicating that the toxin status and the presence or absence of an ulcer do not affect the chemiluminescence response induced by opsonised strains. The presence of *H. pylori* antibody in any sera used for opsonisation did not affect the results.

Discussion

From our study, 66.7% non-opsonised strains of *H. pylori* from peptic ulcer patients induced cell vacuolation, which is significantly higher than those from patients without confirmed peptic ulcer (29%). This supports a previous report that the cytotoxin producing strains were more frequently found in patients with peptic ulcer.¹¹ The mean titre of toxin produced in vitro was also higher in those strains from peptic ulcer patients than that from patients with chronic gastritis only (22.7 versus 7.7). Using a two way analysis of variance approach, we investigated the effect of toxinogenicity and the presence or absence of peptic ulcer on the oxidative burst induced by non-opsonised strains of *H. pylori*. It is found that cytotoxin producing strains have an increased chemiluminescence response in terms of higher peak count and shorter time to reach peak than those toxin negative strains from patients without peptic ulcer. These results support a possible link between the neutrophil respiratory burst and the toxinogenicity of strains of *H. pylori*. The association of certain strains with peptic ulcer disease in itself did not influence significantly the chemiluminescence response of human PMN. However, this finding does not exclude a possible relation between the neutrophil respiratory burst and peptic ulceration, as most strains from peptic ulcer patients (66.7% in this study) are cytotoxin producing. Also we cannot rule out the possibility that some chronic gastritis patients infected with a cytotoxin producing strain might have had an ulcer in the past or may develop one in the future.

Most opsonised strains of *H. pylori* gave an increased chemiluminescence response in our study and the serum antibody to *H. pylori* showed no effect on the chemiluminescence response. This is consistent with previous reports,^{17, 18} which suggested that the serum

TABLE II Chemiluminescence response induced by two representative strains of *H. pylori* in three experiments. Strain 5617B induced a higher and quicker chemiluminescence response. 7994K induced a weaker and slower one

Strains	Test	Peak value (kcps)	Peak time (min)
5617B	1	534	15
	2	490	15
	3	458	16
		494 (38.2)*	
7994K	1	118	39
	2	90	40
	3	71	40
		93 (23.6)*	

*Mean (SD).

TABLE III Peak count and time to reach peak in the chemiluminescence response induced by 76 strains of *H pylori* in relation to the toxinogenicity and the presence or absence of peptic ulcer disease (where indicated, $p < 0.05$, otherwise not significant)

Groups	Characteristic	Number	Means (SD)	
			Peak count (kcps)	Time (min)
1	Tox+ Ulcer+	30	295.1 (128.1)*	18.8 (9.86)‡
2	Tox+ Ulcer-	9	346.2 (193.9)†	21.1 (11.2)§
3	Tox- Ulcer+	15	245.3 (139.8)	25.6 (12.8)
4	Tox- Ulcer-	22	175.7 (116.7)	31.1 (11.3)

* $p < 0.05$ with a 95% CI (38.6, 200.6) for difference in means between group 1 and 4. † $p < 0.05$, 95% CI (5.7, 335.3) between 2 and 4. ‡ $p < 0.05$, 95% CI (-19.6, -5.2) between 1 and 4. § $p < 0.05$, 95% CI (-19.2, -0.9) between 2 and 4. (Two way analysis of variance and multiple comparison).

TABLE IV Peak count and time to reach peak in the chemiluminescence response induced by 42 opsonised strains of *H pylori* in relation to the toxinogenicity and the presence or absence of peptic ulcer

Groups	Characteristic	Number	Means (SD)	
			Peak count (kcps)	Time (min)
1	Tox+ Ulcer+	15	522.1 (165.7)	13.4 (3.7)
2	Tox+ Ulcer-	7	504.3 (168.4)	13.4 (3.6)
3	Tox- Ulcer+	10	472.5 (128.4)	13.5 (3.8)
4	Tox- Ulcer-	10	440.2 (159.6)	14.5 (4.7)

* $p > 0.05$, two way analysis of variance.

complement rather than serum antibody is the main opsonin in this respect. However, it is not certain whether complement mediated opsonisation occurs within the gastric mucosa during *H pylori* infection. A few opsonised strains gave a decreased chemiluminescence response than non-opsonised. The reason for this is not known. There is a possibility that some serum proteins may act as electron scavengers.¹⁹

Current studies from other laboratories suggest that there are some differences between strains of *H pylori* in their ability to produce cytotoxin and CagA protein and to cause peptic ulcer disease.²⁰ Although host factors play a very important part in the genesis of peptic ulcer, there is increasing evidence from molecular biology and immunology for the division of *H pylori* strains into at least two major groups – one producing the CagA protein and cytotoxin (type I) and one that does not produce these antigens (type II).²¹ Our study here also showed there is strain to strain variation in the ability to produce cytotoxin, and to induce an oxidative burst in PMNs.

Previous studies showed that CagA/cytotoxin positive strains of *H pylori* are associated with the infiltration of neutrophils in gastric mucosa^{16 20} and also related to an increased expression of interleukin 8, which is a novel cytokine capable of activating neutrophils.²² Based on these results and the findings in this study that indicate a positive association between the neutrophil respiratory burst and the cytotoxicity of *H pylori*, which is also associated with peptic ulceration, it seems, therefore, cytotoxin/CagA positive strains may induce a more aggressive inflammatory response through secretion of toxic component(s), production of cytokines by gastric mucosal cells, recruitment of neutrophils, release of free oxygen radicals, and subsequently cause tissue damage, which may lead to peptic ulceration.

There were some strains that did not produce cytotoxin but could induce a high chemiluminescence response, raising the possibility that some *H pylori* product(s) other than cytotoxin may be important in this respect. So far the nature of the component(s) of *H pylori* that activates neutrophils is inconclusive. Several reports suggested that the activity is mainly caused by a protein(s), although some non-protein molecules such as lipopolysaccharides may also be implicated.^{13 23} Whether the cytotoxin itself (or some other factor) is responsible needs further studies. There is a possibility that the gene encoding for the protein that activates PMNs is coexpressed on the so called type I strains that produce cytotoxin and CagA protein.

In conclusion, toxinogenicity of *H pylori* strains seems to be correlated with free oxygen radical formation by neutrophils and peptic ulceration. The ability of some *H pylori* strains to produce cytotoxin and to induce an oxidative burst of neutrophils may be important in the pathogenesis of peptic ulceration.

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