CagA/cytotoxic strains of *Helicobacter pylori* and interleukin-8 in gastric epithelial cell lines

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Abstract

Aims—To investigate: (1) whether *Helicobacter pylori* directly induces interleukin-8 (IL-8) message expression and protein secretion in established gastric epithelial cell lines; and (2) if CagA/cytotoxin positive and negative strains of *H pylori* differ in their ability to induce epithelial IL-8.

Methods—Gastric epithelial cell lines were co-cultured with *H pylori* NCTC 11637 and 10 clinical isolates (four cytotoxic, six non-cytotoxic) and secreted IL-8 was measured by enzyme linked immunosorbent assay (ELISA). Specific induction of gastric epithelial IL-8 mRNA was examined by reverse transcription and polymerase chain reaction (RT-PCR) amplification.

Results—*H pylori* (NCTC 11637) induced IL-8 secretion from three gastric epithelial cell lines (KATO-3, ST42, AGS) but not from MKN 45 (gastric) or intestinal (SW480, HT29) cell lines. *H mustelae* did not stimulate IL-8 secretion from KATO-3, ST42, and AGS cells. *H pylori* induced IL-8 secretion was reduced by heat killing, sonication, freeze thawing or formalin fixation of the bacteria. CagA/cytotoxin positive strains of *H pylori* induced significantly higher IL-8 secretion than CagA/cytotoxin negative strains in the three positive gastric epithelial cell lines (KATO-3, ST42: $p < 0.01$; AGS: $p < 0.02$). A significant increase ($p < 0.01$) in the expression of IL-8 mRNA relative to G3PDH mRNA was observed in KATO-3 cells after three hours of co-culture with CagA/cytotoxin positive strains.

Conclusions—*H pylori* directly increases gastric epithelial IL-8 mRNA expression and IL-8 protein secretion in a strain specific manner. Induction of epithelial IL-8 by CagA/cytotoxin positive strains is likely to result in neutrophil chemotaxis and activation and thus mucosal damage. These observations on epithelial IL-8 may explain the association between CagA/cytotoxin positive strains and gastroduodenal disease.

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Helicobacter pylori infection is strongly associated with polymorphonuclear cell infiltration into the gastric mucosa.¹ This cellular response probably represents a primary immune defence mechanism against a micro-

bial pathogen. However, infection with *H pylori* is chronic, and while neutrophils can effectively phagocytose *H pylori* in vitro,² there is little evidence that host defence mechanisms eradicate infection in vivo. Once activated, neutrophils have considerable potential for releasing proteolytic enzymes which may damage mucosal integrity.³

H pylori produces various factors which will attract or activate neutrophils in vitro.^{2,4-7} Infection with *H pylori*, however, also results in increased gastric mucosal production of interleukin-8 (IL-8),⁸ a cytokine which is a potent activator and chemotactic agent for neutrophils.⁹⁻¹¹ IL-8 has recently been implicated in the pathogenesis of several inflammatory or infectious conditions.¹²⁻¹⁶ In gastritis the in vitro mucosal production of IL-8 is substantially increased in active gastritis where polymorphs infiltrate into the epithelial layer.⁸ As gastric epithelial cells express IL-8,¹⁷ they may have an important role in regulating primary host defence mechanisms and be functionally involved in the neutrophilic response to *H pylori* infection. In common with other epithelial cells, gastric epithelial production of IL-8 is regulated by cytokines.¹⁸ Bacterial factors may also be important. Studies of urinary epithelial cell lines have recently shown that adherent *Escherichia coli* will induce IL-8 secretion.¹⁹

Recent studies have shown that a mucosal IgA response to the 120 kilodalton protein of *H pylori* is strongly associated with polymorph infiltration into the gastric epithelium.²⁰ This high molecular weight protein, which is strongly associated with bacterial cytotoxicity,²¹⁻²³ has recently been termed the CagA protein following cloning of the gene.^{24,25} The CagA gene is nearly always absent from non-cytotoxic strains,^{24,25} so mucosal IgA recognition of the gene product is likely to reflect the characteristics of the colonising strain(s). The linking of the mucosal IgA response to the CagA protein and polymorph infiltration could reflect differences in induction of IL-8.

In this study we investigated whether *H pylori* directly induces IL-8 secretion from gastric epithelial cell lines and examined whether cytotoxic strains expressing the CagA protein differed from CagA/cytotoxin negative strains in their ability to induce epithelial IL-8 mRNA expression and IL-8 protein secretion.

Methods

GASTROINTESTINAL EPITHELIAL CELL LINES
Four gastric epithelial cell lines KATO-3 (European Collection of Animal Cell Cultures

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(ECACC), Salisbury, Wiltshire), AGS (ECACC), MKN 45²⁶ and ST42²⁷ (kindly provided by Dr S Watson) were used. Intestinal cell lines HT29 (ECACC) and SW480 (ECACC) were used as controls. Cell lines were routinely maintained in RPMI 1640 (ICN-Flow Laboratories, High Wycombe, Bucks) supplemented with 10% heat inactivated fetal calf serum (FCS) (Sera Lab, Crawley, Surrey) and 40 µg/ml gentamicin. Initial studies had shown that all six lines secreted IL-8 in response to IL-1β (gift of Glaxo Group Research, UK). The intestinal cell lines HT29²⁸ and SW480 were selected as controls because they secreted high IL-8 concentrations in response to cytokines (HT29, SW480) and adherent *E coli* (SW480) (unpublished observations).

BACTERIA

The type strain of *H pylori* NCTC 11637 (CagA/cytotoxin positive) and 10 clinical isolates were used. Six strains (G12, G17, G21, G25, G47, G50) were non-cytotoxic and four strains (G27, G32, G39, G65) were cytotoxic. Western blotting of whole cell bacterial preparations with CagA antibody positive human sera showed that the cytotoxic strains all expressed the high molecular weight CagA protein and that this protein was not expressed in the non-cytotoxic strains. Two strains of *H mustelae* (NCTC 12031 and NCTC 12032, both CagA/cytotoxin negative) were used as controls. Bacteria were grown on blood agar base Number 2 (Oxoid, Basingstoke, Hants) incorporating 7% fresh horse blood and harvested on day 4 into phosphate buffered saline (PBS) and used immediately.

In some experiments *H pylori* NCTC 11637 were heat killed (30 minutes, at 80°C), sonicated, freeze-thawed or fixed in 0.5% formalin (30 minutes). Formalin fixed bacteria were washed three times in PBS and resuspended in culture medium. Ultracentrifuged (100 000 × *g* for one hour) supernatant fluids of sonicated NCTC 11637 were also used at a concentration of 10 µg/ml.

BACTERIAL-EPITHELIAL CO-CULTURE

EXPERIMENTS

Epithelial cells were plated into 24 well plates (ICN-Flow) at a density of 1 × 10⁵/ml and cultured for three days to confluence (about 5 × 10⁵/ml). Bacteria were washed in RPMI 1640 containing 10% FCS without gentamicin and resuspended at a concentration of 2.5 × 10⁷/ml. Epithelial cells were cultured alone or with bacterial preparations for 24 hours at 37°C in a 95% air and 5% CO₂ humidified incubator. At the end of culture supernatant fluids were aspirated and frozen at -70°C until assayed and cell viability determined by trypan blue exclusion. No differences in epithelial cell viability in experimental and control cultures without *H pylori* were evident.

The carboxylic ionophore monensin (Sigma, Poole, Dorset), which has been shown to block cytoplasmic vacuolisation

induced by *H pylori* cytotoxin,²⁹ was included in some experiments at concentrations of 2–10 µg/ml. The effect of monensin on *H pylori* induced IL-8 secretion was compared with IL-1β induced IL-8 secretion.

IL-8 ELISA

IL-8 concentrations in culture supernatant fluids were assayed by enzyme linked immunosorbent assay (ELISA) as described before,⁸ using a murine monoclonal antibody to IL-8 and a phosphatase conjugated goat anti-IL8 polyclonal antibody. Culture supernatant fluids were diluted 1 in 4 in PBS/0.1% Tween containing 1% bovine serum albumin (BSA). Concentrations of IL-8 were determined from a standard curve (62.5 pg/ml–2000 pg/ml) of recombinant IL-8 (Sandoz, Vienna, Austria). Bacterial induced IL-8 secretion was expressed as ng/ml following subtraction of background unstimulated control culture values.

IL-8-mRNA

The effects of CagA/cytotoxin positive and CagA/cytotoxin negative *H pylori* on epithelial IL-8 mRNA expression in KATO-3 cells were examined. After three hours of bacterial co-culture, total RNA from KATO-3 cells was extracted using RNeasy (Qiagen Ltd, Crawley, Sussex). Briefly, cells were pelleted and resuspended in 1 ml RNeasy with 0.1 ml chloroform, the phases separated, and RNA precipitated from the aqueous phase with an equal volume of isopropanol. The ethanol washed pellets were then resuspended in 20 µl water containing RNase inhibitor. Messenger RNA was reverse transcribed using MMLV-RT primed with oligo dT15. The cDNA was PCR amplified for 20 cycles with primers specific for IL-8¹⁷ and an internal control gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH). To determine the ratio of IL-8 mRNA to G3PDH mRNA, ³²P-dATP (Amersham, Bucks, UK) was added to the PCR reaction and the ratio of incorporation of radiolabel into IL-8 and G3PDH PCR products quantified by scintillation counting. Results are expressed as a mean of five determinations.

Data are expressed as mean (SEM). Differences in IL-8 secretion and IL-8 mRNA expression following co-culture with cytotoxic and non-cytotoxic strains were examined using the Mann-Whitney U test.

Results

No detectable IL-8 was secreted by unstimulated MKN 45, HT29, or SW480 cell lines over 24 hours. In the KATO-3, ST42, and AGS cell lines 24 hour IL-8 secretion from unstimulated cells ranged from 0–2.38, 0–1.38, and 0–0.24 ng/ml, respectively. The 24 hour secretion of IL-8 from all six cell lines following co-culture with the *H pylori* type strain NCTC 11637 is shown in table 1. AGS, KATO-3, and ST42 cells all secreted IL-8 on co-culture with *H pylori* but bacterial co-culture induced minimal or no IL-8

Table 1 Mean (SEM) IL-8 secretion by gastrointestinal cell lines after 24 hour culture with *H. pylori* strain NCTC 11637 or *H. mustelae* strain NCTC 12032

Cell line	IL-8 secretion (ng/ml)	
	<i>H. pylori</i>	<i>H. mustelae</i>
KATO-3	5.1 (0.53)	0
ST42	2.8 (0.81)	0
AGS	3.9 (0.14)	0
MKN45	0	ND
HT29	0	ND
SW480	0.24 (0.13)	ND

ND = not done; n = 5.

Table 2 Effects of heat killing, freeze-thawing, sonication and formalin fixation on *H. pylori* (NCTC 11637) induced IL-8 secretion by ST42 gastric epithelial cells

	NCTC 11637	(Mean SEM) IL-8 secretion (ng/ml)
(A) Heat killed	0.86 (0.08)*	
Freeze-thawed	0.81 (0.08)*	
Sonicated	0.74 (0.17)*	
No treatment (matched controls)	2.5 (0.44)	
(B) Formalin fixed	0.66 (0.03)*	
No treatment (matched controls)	3.59 (0.27)	

*p < 0.02 from untreated controls; n = 5.

Figures 1A-C IL-8 secretion (net) from KATO-3 (A), ST42 (B), and AGS (C) gastric epithelial cells after 24 hours of co-culture with *CagA*/cytotoxin positive and *CagA*/cytotoxin negative strains of *H. pylori*. Values are mean (SEM) (n = 5).

secretion from MKN 45 gastric cells or HT29 and SW480 intestinal cells. Neither of the two strains of *H. mustelae* induced IL-8 secretion from the three human gastrointestinal epithelial cell lines producing IL-8 in response to *H. pylori* (table 1).

The effects of heat killing, sonication, and freeze-thawing of *H. pylori* on bacterial induced IL-8 secretion from the ST42 epithelial cell line are shown in table 2. A significant reduction ($p < 0.02$) in IL-8 secretion was apparent if non-viable bacterial preparations were used. Formalin fixation of *H. pylori* also significantly reduced ($p < 0.02$) 24 hour IL-8 secretion by ST42 cells compared with unfixed paired control cultures of *H. pylori* (table 2). No secretion of IL-8 was observed in the three cell lines after co-culture with ultracentrifuged supernatant fluids at a concentration of 10 $\mu\text{g/ml}$.

IL-8 production from KATO-3, ST42, and AGS gastric epithelial cell lines after 24 hours of co-culture with *CagA*/cytotoxin positive and negative strains of *H. pylori* is shown in figs 1A-C. In all three cell lines the *CagA*/cytotoxin positive strains induced significantly higher IL-8 secretion than the *CagA*/cytotoxin negative strains (KATO-3, ST42: $p < 0.01$; AGS: $p < 0.02$).

The effects of monensin on *H. pylori* (NCTC 11637) and IL-1 β induced IL-8 secretion by ST42 cells are shown in table 3. No reduction in epithelial cell viability or stimulation of IL-8 secretion was observed in controls cultured with monensin alone. Monensin at 2, 5, and 10 $\mu\text{g/ml}$ significantly reduced the 24 hour IL-8 production induced by *H. pylori* ($p < 0.02$, 10 $\mu\text{g/ml}$; $p < 0.05$, 5 $\mu\text{g/ml}$; $p < 0.02$, 2 $\mu\text{g/ml}$) (table 3). A significant reduction ($p < 0.01$) in IL-1 β (10 ng/ml) induced IL-8 production by ST42 cells was also observed on co-culture with monensin. Monensin similarly inhibited both *H. pylori* and IL-1 β induced IL-8 secretion in the AGS and KATO-3 cell lines (data not shown).

Figure 2 PCR analysis of mRNA using primers specific for IL-8 (369 base pairs) and G3PDH (157 base pairs) in KATO-3 gastric epithelial cells after three hours of co-culture with *H. pylori*. Cell only control (lane 1), *CagA*/cytotoxin negative strains G25 and G50 (lanes 2 and 3), *CagA*/cytotoxin positive strains G32 and NCTC 11637 (lanes 4 and 5), 100 base pair ladder (Gibco-BRL) (lane L).

Table 3 Effects of monensin on *H pylori* (NCTC 11637) and IL-1 induced IL-8 secretion from ST42 gastric epithelial cells

Monesin μ g/ml IL-8 ng/ml	<i>H pylori</i> (NCTC 11637)				IL-1 β (10 ng/ml)	
	0	2	5	10	0	10
	3.58 (0.27)	2.09 (0.08)**	2.45 (0.22)*	2.32 (0.13)**	6.25 (0.46)	3.29 (0.27)**

Values are mean (SEM), n = 5.
Mann-Whitney U test: *p < 0.05; **p < 0.02; ***p < 0.01.

Table 4 Effect of *H pylori* CagA/cytotoxin positive and CagA/cytotoxin negative strains on IL-8 mRNA in KATO-3 cells following three hours of co-culture

	Cell only control	CagA/cytotoxin positive strains		CagA/cytotoxin negative strains	
		NCTC 11637	G32	G50	G25
IL-8:G3PDH PCR products	0.19 (0.02)	0.60 (0.09)***	0.74 (0.18)***	0.22 (0.04)	0.30 (0.06)

Results expressed as a ratio of IL-8 to G3PDH PCR products, values are mean (SEM), n = 5.
Mann-Whitney U test: ***p < 0.01.

Using a semiquantitative PCR technique, the relative induction of IL-8 mRNA in KATO-3 cells was compared with expression of G3PDH mRNA. At three hours there was a significant increase (p < 0.01) in IL-8 PCR product in the KATO-3 cells following co-culture with the CagA/cytotoxic strains (NCTC 11637 and G32) relative to unstimulated cells (fig 2, table 4). In contrast, co-culture with the CagA/cytotoxic negative strains (G25 and G50) resulted in no increase in IL-8 PCR product (fig 2, table 4).

Discussion

It is now clear that human gastric epithelial cells express IL-8 in vivo and IL-8 expression is increased in *H pylori* gastritis.¹⁷ Studies with gastric epithelial cell lines have shown that epithelial derived IL-8 is not only immunologically active but also biologically active inducing neutrophil migration¹⁸ and activation.³⁰ The gastric epithelium therefore has the potential for an active role in host mucosal defences. The present study clearly shows that *H pylori* directly increases gastric epithelial cell IL-8 mRNA expression and IL-8 protein secretion in a strain specific manner. The strain specificity of this response has important implications for the pathogenesis of *H pylori* related gastroduodenal disease.

The gastric epithelium represents the primary interface with *H pylori*. In acute gastritis the initial histopathological response is neutrophilic³¹ and rapid secretion of IL-8 by the epithelium may represent a primary host defence mechanism. The epithelial IL-8 response to *H pylori* observed in this study was restricted to gastric cell lines, suggesting a specific host-bacterial interaction related to the specificity of colonisation within the gastroduodenal tract. Neither of the two intestinal cell lines, which are capable of secreting high concentrations of IL-8, produced IL-8 in response to *H pylori*. It will be interesting to examine the IL-8 responses of epithelial cells from non-gastrointestinal sites to *H pylori* to investigate further the specificity of this response. Recent studies have shown that cervical³² and bronchial³³ epithelial cells will

produce IL-8 in response to a range of bacterial species.

H pylori induced stimulation of IL-8 from gastric epithelial cell lines was dependent on the presence of viable bacteria, with formalin fixation, heat killing, sonication, and freeze-thawing of organisms all substantially reducing their ability to stimulate IL-8. The dependence on live *H pylori* bacteria suggests that induction of IL-8 requires an active process. Interestingly, recent studies on polymorph transepithelial migration in response to *Salmonella typhimurium* also showed that direct bacterial-epithelial contact and bacterial protein synthesis were essential.³⁴

Recent studies on a range of enteric pathogenic bacteria suggest that IL-8 is secreted from intestinal cells in response to bacterial invasion.³² However, ultrastructural studies on the gastric epithelial cell lines used in this study have shown frequent internalisation of *H pylori* into AGS and ST42 cell lines but minimal invasion of KATO-3 cells (unpublished data). IL-8 stimulation by *H pylori*, whilst requiring viable organisms, is therefore unlikely to relate directly to internalisation, as similar strain specific patterns of IL-8 responses were observed in all three cell lines.

CagA/cytotoxic strains of *H pylori* induced significantly higher degrees of epithelial IL-8 secretion and IL-8 mRNA expression than CagA/cytotoxic negative strains. Our previous studies have identified that mucosal immune recognition of the CagA protein is strongly associated with epithelial polymorph infiltration.²⁰ Increased stimulation of epithelial IL-8 induced by CagA/cytotoxic strains may be critical in inducing enhanced gastric neutrophil migration and activation. Once activated, neutrophils will also secrete IL-8, thus perpetuating the inflammatory response to infection³⁵ and the potential for mucosal damage. The local production of IgA autoantibodies to IL-8, however, may represent a down-regulatory mechanism of the host to block excessive IL-8 induced neutrophil activation.⁸

There is now increasing evidence linking strains of *H pylori* expressing the cytotoxin and associated CagA protein with gastroduodenal disease. Patients with peptic ulcers are

more likely to be infected with strains of *H. pylori* which are cytotoxic.³⁶ Increased mucosal IgA²⁰ and systemic IgG^{21, 22, 37} recognition of the cytotoxin-associated high molecular weight CagA protein is evident in patients with peptic ulcer disease. Patients with gastric cancer also show increased serological recognition of the CagA protein relative to subjects with non-ulcer dyspepsia.³⁸

Whether the consistent association of CagA/cytotoxic *H. pylori* strains with gastroduodenal disease is a consequence of their cytotoxicity is unclear. Serum IgG³⁹ and mucosal IgA⁴⁰ antibodies inhibit cytotoxicity in vitro, suggesting that if a similar phenomenon occurs in vivo, the cytotoxin may not be the primary virulence factor inducing mucosal damage. In the present study monensin, an inhibitor of cytotoxicity in vitro,²⁹ reduced *H. pylori* induced epithelial IL-8 secretion. However, this effect could not be attributed to blocking of a specific bacterial induction mechanism as IL-1 β induced IL-8 secretion was similarly inhibited by monensin.

The monensin induced reduction in IL-8 secretion is presumably a consequence of impairment of Golgi function and intracellular transport.^{41, 42} Ultrastructural studies (unpublished observations) have also shown that at the bacteria:cell ratio used in our study, there was minimal evidence of intracytoplasmic vacuolisation at 24 hours in the three gastric cell lines. In contrast to the cytotoxin, the function of the CagA protein is unknown. This highly immunogenic protein is located on the surface but whether it is involved in epithelial cell adhesion is currently unclear.

Further studies using mutant strains of *H. pylori* should determine the relative roles of the CagA protein and the cytotoxin in the stimulation of gastric epithelial IL-8 production. Whilst epithelial expression of IL-8 will also be regulated by cytokines,¹⁸ with the local production of tumour necrosis factor⁴³ increasing expression, identification of specific *H. pylori* components inducing epithelial IL-8, and the gastric epithelial cell receptors involved, may facilitate the development of novel gastric anti-inflammatory agents.

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