**Helicobacter pylori** induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype

J E Crabtree, A Covacci, S M Farmery, Z Xiang, D S Tompkins, S Perry, I J D Lindley, R Rappuoli

Abstract

**Aims**—To use a range of natural phenotypically variant strains of *Helicobacter pylori* with disparate CagA and VacA (vacuolating cytotoxin) expression to determine which bacterial factors are more closely associated with epithelial interleukin-8 (IL-8) induction.

**Methods**—Gastric epithelial cells (AGS and KATO-3) were co-cultured with five *H pylori* strains which were variously shown to express the cagA gene/CagA protein, VacA and/or to exhibit biological cytotoxicity. Secreted IL-8 was assayed by enzyme linked immunosorbent assay (ELISA) and IL-8 messenger RNA (mRNA) was assayed using a reverse transcription polymerase chain reaction based technique (RT-PCR).

**Results**—Strains expressing CagA, including a variant strain (D931) which is non-cytotoxic and does not express the VacA protein, were found to upregulate epithelial IL-8 secretion and gene expression. In contrast, strains with no CagA expression, even in the presence of VacA and/or biological cytotoxicity, (G104, BA142), failed to induce IL-8 protein or mRNA above control values.

**Conclusions**—These results strongly support a role for *H pylori* CagA or coexpressed factors other than the cytotoxin in upregulation of gastric epithelial IL-8. Increased epithelial IL-8 secretion and concomitant neutrophil chemotaxis and activation in addition to direct cytotoxicity may be an important factor in tissue damage and ulceration.

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The epithelium of the human gastroduodenal mucosa expresses interleukin-8 (IL-8), a potent chemotactic and activating factor for neutrophils. Interleukin-8 may be functionally involved in regulating neutrophil responses to the gastric pathogen *Helicobacter pylori*. Infection with this bacterium is characterised by neutrophil infiltration into the gastric mucosa and epithelial layer and such cellular responses may be important in tissue damage and ulceration. *H pylori* infection is associated with both increased epithelial IL-8 expression and secretion of IL-8. The bacterium will directly upregulate IL-8 messenger RNA (mRNA) expression and IL-8 protein secretion in gastric epithelial cell lines. This response appears to be restricted to epithelial cell lines from gastric sites within the gastrointestinal tract, suggesting a specific host-bacteria interaction related to the specificity of colonisation.

Although *H pylori* shows extreme genomic variability, recent studies have shown that strains can be divided into two major groups, one in which both a 128 kDa antigen (CagA) and a vacuolating cytotoxin (VacA) are expressed, and another which does not produce these proteins. Previous studies have linked bacterial cytotoxicity, mucosal IgA, and systemic IgG recognition of the CagA protein (also defined as a 120–130 kDa antigen), with peptic ulceration strongly suggesting these strains have greater ulcerogenic potential. Although others have argued that there are no ulcerogenic strains of *H pylori* and host responses are critical in ulcerogenesis, induction of epithelial IL-8 by *H pylori* was recently shown to be strain specific. Interestingly, *H pylori* strains expressing the CagA 128 kDa protein and the strongly associated vacuolating cytotoxin induce IL-8 secretion from gastric epithelial cells but minimal secretion occurs after culture with non-cytotoxic CagA negative strains. In this study we have recently identified phenotypic variants of *H pylori* with disparate CagA and cytotoxin expression (Xiang Z et al, 1994, unpublished observations) to investigate which of these two virulence factors is associated with IL-8 induction in two gastric epithelial cell lines.

**Methods**

Five *H pylori* strains were used: *H pylori* NCTC 11637 and four clinical isolates G50, BA142, D931, and G104. Cytotoxic *H pylori* NCTC 11637, which expresses the CagA protein and induces epithelial IL-8 production, and non-cytotoxic CagA negative G50 were the positive and negative controls, respectively. The presence of the cagA gene was determined by the polymerase chain reaction (PCR) using cagA gene specific primers and by Southern blotting using two fragments of the cagA gene spanning nucleotides 521 to 1840 and nucleotides 1460 to 2620 as probes. CagA protein expression was characterised by western blotting using mouse polyclonal antiserum raised against electroeluted antigen isolated from NCTC 11637 bacterial extracts and rabbit antiserum to the recombinant fragment 17/12 of CagA. The fusion protein was

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STIMULATION OF GASTRIC EPITHELIAL CELL LINES WITH H PYLORI

Two gastric epithelial cell lines KATO-3 (European Collection of Animal Cell Cultures (ECACC), Salisbury, Wiltshire) and AGS (ECACC) were routinely maintained in RPMI 1640 supplemented with 10% FCS and 40 μg/ml gentamicin. AGS cells were plated into 24 well plates (ICN-Flow) at a density of 1 × 10⁵/ml and cultured for three days to confluence (approximately 5 × 10⁵/ml). KATO-3 cells, which are non-adherent, were resuspended in RPMI 1640 containing 10% FCS without gentamicin and used at a final concentration of 5 × 10⁵/ml. After removal of the gentamicin containing media from AGS monolayers, gastric epithelial cells (AGS and KATO-3) were cultured in quadruplicate either alone or with bacterial preparations for 24 hours at 37° C in a 95% air, 5% CO₂ humidified incubator. Supernatants were aspirated at 24 hours and stored at −70° C until assayed using an enzyme linked immunosorbent assay (ELISA). In parallel experiments KATO-3 cells were cultured with H pylori strains for three hours for examination of cellular IL-8 messenger RNA (mRNA) expression.

IL-8 ELISA

Interleukin-8 in cell culture supernatants was assayed in duplicate by ELISA as described previously using a murine monoclonal antibody to IL-8 and a phosphatase conjugated goat anti-IL8 polyclonal antibody. Concentrations of IL-8 were determined from a standard curve of recombinant IL-8 (Sandoz, Vienna, Austria) and concentrations were expressed as ng/ml. The detection limit of the ELISA was 62 pg/ml.

IL-8 mRNA EXPRESSION

Interleukin-8 mRNA expression was determined as described previously. Briefly, after three hours of bacterial co-culture, total RNA from KATO-3 cells was extracted using RNAzolB (Biogenesis Ltd, Bournemouth) and mRNA was reverse transcribed using MMLV reverse transcriptase primed with oligo dT15. The complimentary DNA was generated by cloning the region of the cagA gene between nucleotides 2776 and 3467 into the MS2 polymerase gene carried in the pEX 34B plasmid vector. Water extracts of bacterial cells were assessed for cytotoxicity against HeLa cells in vitro, as previously described. The presence of the VacA protein was determined by western blotting and rabbit antiserum specific for recombinant VacA.

Epithelial co-culture experiments were undertaken without prior knowledge of the phenotypic characteristics of the variant strains. For these experiments, bacteria were grown on blood agar base Number 2 (Oxoid, Basingstoke, Hampshire) including 7% fresh horse blood. Bacteria were harvested on day 4 into phosphate buffered saline (PBS). After centrifugation, bacteria were resuspended at 2.5 × 10⁹/ml in RPMI 1640 (ICN-Flow Laboratories, High Wycombe, Bucks) supplemented with 10% heat inactivated fetal calf serum (FCS) (Sera Lab, Crawley, Surrey) and used immediately.

Figure 1 PCR amplification of cagA gene (C) and urease gene A (U) (PCR control) in the H pylori NCTC 11637 (lanes 1 and 2), D931 (lanes 3 and 4), BA142 (lanes 5 and 6), G104 (lanes 7 and 8), and G50 (lanes 9 and 10) strains. Lanes 11 and 12 contain DNA negative controls; lane L contains a base pair ladder (Gibco-BRL). NCTC 11637 and D931 are positive for the cagA gene.
amplified using PCR for 20–30 cycles with primers specific for IL-8 and an internal control gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH). 32P-dATP (Amersham, Bucks) was added to the PCR reaction and the ratio of incorporation of radiolabel into IL-8 and G3PDH PCR products quantified using scintillation counting.

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

Results

H pylori strain phenotype

The phenotype of the three variant strains, together with the positive (NCTC 11637) and negative (G50) controls is shown in the table. One of the variant strains, D931, which was non-cytotoxic and did not express VacA, was positive for the cagA gene on PCR (fig 1) and Southern blotting, and expressed the CagA protein (fig 2). Strains G104 and BA142 showed no expression of the cagA gene on PCR (fig 1) or Southern blotting, and did not express the CagA protein (fig 2), although both strains showed biological cytotoxicity and G104 expressed the VacA protein (table). The absence of VacA protein in western blots of the biologically cytotoxic BA142 strain may reflect disparity between immunoreactive and biologically functional epitopes.

Effects of H pylori strains on IL-8 secretion

Secretion of IL-8 by KATO-3 and AGS gastric epithelial cell lines over the 24 hours following stimulation with H pylori strains is shown in figs 3A and B, respectively. In KATO-3 cells significant secretion of IL-8, compared with cell only control cultures, was observed after stimulation with the VacA positive/CagA positive NCTC 11637 strain (p < 0·01) and the CagA positive/VacA negative D931 strain (p < 0·02) (fig 3A). The CagA negative/VacA negative G50 strain and cytotoxic CagA negative strains, G104 and BA142, did not induce secretion of IL-8 from KATO-3 cells. In KATO-3 cells IL-8 secretion induced by NCTC 11637 and D931 was significantly greater (p < 0·05) than that induced by G50, BA142, and G104. In the AGS cell line, as previously observed,7 the NCTC 11637 strain induced lower levels of IL-8 secretion than in the KATO-3 cells (fig 3B). In AGS cells significant IL-8 secretion, when compared with cell only controls, was observed after co-culture with NCTC 11637 and D931 (p < 0·02) but not G50, BA142, and G104 (fig 3B).

Effects of H pylori strains on IL-8 mRNA expression in KATO-3 cells

Initial time course studies revealed optimum IL-8 mRNA expression in KATO-3 cells three hours after stimulation with H pylori. Figure 4 shows the ratio of IL-8 mRNA to G3PDH PCR products in KATO-3 cells three hours after stimulation with the five H pylori strains. A significant increase in the ratio of IL-8 to G3PDH PCR products was evident after culture with NCTC 11637 (p < 0·01) and D931 (p < 0·05) compared with cell only controls. Stimulation of KATO-3 cells with strains G50, G104, and BA142 did not result in an increase in the IL-8 to G3PDH ratio (figs 4 and 5).

Discussion

The induction of epithelial IL-8 production by H pylori occurs in a strain specific manner,7 with those strains expressing the CagA protein and the strongly associated vacuolating cytotoxin stimulating IL-8 secretion. As CagA and cytotoxin expression are very closely associated,9–11 our previous studies could not
Figure 4 Effects of H pylori strains on IL-8 mRNA expression in KATO-3 gastric epithelial cells. The results show the mean (SEM) ratio of IL-8 to G3PDH PCR products (n = 5) three hours after culture with G50, NCTC 11637, G104, BA142, and D931. CT ± = strains with or without cytotoxic bioactivity. * p < 0.05, ** p < 0.01.

determine which bacterial factor was more closely linked with IL-8 induction. However, monensin, a carboxyl ionophore, which blocks cytoplasmic vacuolisation induced by H pylori cytotoxin,33 did not specifically inhibit epithelial IL-8 induction,7 suggesting other bacterial factors associated with cytotoxin expression may be important in upregulating IL-8 secretion.

The role of the highly immunogenic CagA protein, which shows size heterogeneity,9 in the pathogenesis of H pylori infection is unclear. Earlier studies have shown that non-cytotoxic strains almost invariably lack the cagA gene, suggesting that the CagA protein may be necessary either for transcription or production of a functional cytotoxin.9,10 However, recent molecular screening of a large number of strains has identified naturally occurring phenotypic variants in which the CagA and VacA proteins are not co-expressed (Xiang Z et al, 1994, unpublished observations), suggesting that cagA gene expression is not essential for functional cytotoxicity. In contrast to cagA, the vacA gene is present in all strains of H pylori; however, the gene is silent in non-cytotoxic strains which do not secrete the 94 kDa active VacA protein.11 BA142, although biologically cytotoxic, did not possess the immunoreactive VacA protein. This may reflect either a disparity between biological and immunological functional epitopes in this strain, or the synthesis of a labile variant of the vacuolating cytotoxin. Additional data supporting the latter hypothesis is that northern blot analysis of mRNA from BA142 shows that the vacA gene is actively transcribed in this strain.

In the present study using the natural phenotypic variants of H pylori, we show that CagA expression, and not VacA expression, is associated with upregulation of epithelial IL-8 mRNA expression and stimulation of IL-8 protein secretion. In both AGS and KATO-3 epithelial cell lines the CagA positive/cytotoxin negative D931 strain increased IL-8 mRNA expression and induced IL-8 protein secretion, albeit to lower levels than NCTC 11637. Cytotoxic strains lacking the cagA gene did not induce IL-8. Quantitative differences in CagA expression, or co-expressed surface factors, may account for differing levels of IL-8 induction.

The lack of induction of IL-8 by CagA negative cytotoxic strains is of particular interest as oral administration of purified cytotoxin (VacA) to mice induces gastric epithelial lesions and cell necrosis but not inflammatory cell infiltration.12 Infection with cytotoxic strains of H pylori, however, has recently been associated with increased polymorph infiltration in human antral mucosa.13 As epithelial IL-8 induction does not relate to cytotoxin expression, this observation may be secondary to expression of other bacterial factors. Our previous studies have demonstrated a strong association between mucosal IgA recognition of the CagA protein and antral polymorph infiltration.14 Induction of gastric epithelial IL-8 by the CagA protein, or co-expressed surface factors other than the cytotoxin, may be an important factor regulating neutrophil chemotaxis and may explain these histopathological observations.

Both host and bacterial factors are important for determining the extent of mucosal cellular responses to H pylori infection. Bacterial products25-29 and a range of inflammatory mediators such as C5a, leukotriene B4, and platelet activating factor,30 in addition to IL-8, may also regulate polymorph infiltration and activation in response to H pylori infection. Previous studies have linked both immunological recognition of the CagA protein24-26 and bacterial cytotoxicity27 with peptic ulceration. The inhibition of in vitro cytotoxicity by gastric H pylori specific IgA antibodies38 suggests that the cytotoxin may not be the only factor involved in the pathogenesis of peptic ulceration.
Neutrophils are strongly linked with peptic ulceration and may be important mediators of mucosal damage. Interleukin-8, and related members of the chemokine family, have been implicated in the pathogenesis of several inflammatory and infectious conditions characterised by neutrophil infiltration and the importance of the epithelium, the primary host–bacteria interface, as a source of immunoregulatory peptides is becoming increasingly recognised.

Differential induction of IL-8 in gastric epithelial cells by *H pylori* strains may be an important factor determining the cellular response to infection.

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