Chemokine mRNA expression in gastric mucosa is associated with *Helicobacter pylori* cagA positivity and severity of gastritis

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Abstract

***Aim*—To investigate the association between the quantity of gastric chemokine mRNA expression, severity of gastritis, and cagA positivity in *Helicobacter pylori* associated gastritis.**

***Methods*—In 83 dyspeptic patients, antral and corpus biopsies were taken for semiquantitative reverse transcription polymerase chain reaction (RT-PCR) and histological grading of gastritis. Gastritis was evaluated by visual analogue scales. Quantities of chemokine (IL-8, GROα, ENA-78, RANTES, MCP-1) RT-PCR products were compared with G3PDH products. Each sample was also evaluated for the presence of cagA and ureA mRNA by RT-PCR.**

***Results*—mRNA expression of all five chemokines was significantly greater in *H pylori* positive than in *H pylori* negative mucosa. In *H pylori* positive patients, in the antrum C-X-C chemokine mRNA expression was significantly greater in cagA positive patients than in cagA negative patients, but there were no significant differences in C-C chemokine mRNA expression. In *H pylori* positive patients, chemokine mRNA expression in the corpus was less than in the antrum. In contrast to the antrum, only GROα mRNA expression was significantly greater in cagA positive infection. Polymorphonuclear cell infiltration was correlated with C-X-C chemokine mRNA expression. Significant correlations were also found between bacterial density and C-X-C chemokine mRNA expression.**

***Conclusions*—In *H pylori* infection, C-X-C chemokines may play a primary role in active gastritis. Infection with cagA positive *H pylori* induces greater gastric chemokine mRNA expression in the antral mucosa, which may be relevant to the increased mucosal damage associated with cagA positive *H pylori* infection.**

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Many studies have implicated infection with *Helicobacter pylori* in the pathogenesis of chronic gastritis. Histologically, the host response to *H pylori* infection is characterised by infiltration of lymphocytes, macrophages, and neutrophils into the gastric mucosa.[1] In addition, various inflammatory mediators have been detected in higher concentrations in infected gastric mucosa. Chemokines are a recently described family of inflammatory cytokines that have leucocyte chemotactic and activating properties.[2–4] The chemokine superfamily has been divided into two major subgroups: the C-X-C and the C-C chemokines. These two subfamilies differ in their biological properties. In general, the C-X-C chemokines seem to affect primarily neutrophils, while the C-C chemokines have functional action on monocytes and lymphocytes.[2 3] These selective chemoattractant activities of chemokines play a major role in regulating leucocyte populations migrating into tissues.

Previous studies have shown that *H pylori* infection is associated with increased gastric mucosal interleukin-8 (IL-8).[5–7] *H pylori* strains expressing the cytotoxin associate gene A (cagA) have been associated with increased gastric mucosal IL-8 in vivo[8 9] and with induction of IL-8 in gastric epithelial cell lines in vitro.[10–12] The cagA gene is part of the cag pathogenicity island (PAI) and is not the direct inducer of IL-8,[11 12] but multiple genes in the cag PAI are essential for induction of epithelial chemokines.[13 14] While there have been many studies on IL-8 in the gastric mucosa, other members of the chemokine family have not been fully investigated. Studies to date have not quantified mRNA expression of chemokines, and the relation to gastric histopathology has not been evaluated. In this study, using semiquantitative reverse transcription polymerase chain reaction (RT-PCR), we have investigated the gastric mRNA expression of three C-X-C chemokines (IL-8, growth related oncogene alpha (GROα), epithelial neutrophil activating protein 78 (ENA-78)) and two C-C chemokines (**R**egulated on **A**ctivation **N**ormal **T** cell **E**xpressed and **S**ecreted (RANTES) and monocyte chemotactic protein-1 (MCP-1)). We have examined the association between chemokine mRNA expression, severity of gastritis, and presence of the cagA positive *H pylori* infection.

Methods

PATIENTS

Patients with dyspeptic symptoms scheduled for upper gastrointestinal endoscopy were prospectively enrolled into the study. Patients were excluded if they were over 70 years old, were receiving steroids and non-steroidal anti-inflammatory drugs, or had received anti-ulcer agents or antibiotics during the two months before the examination. All subjects provided

Table 1 Oligonucleotide primers for reverse transcriptase polymerase chain reaction

Gene		Primer	Expected product size
G3PDH	sense	GAGTCAACGGATTTGGTCGT	178
	antisense	TTCCCGTTCTCAGCCTTGAC	
IL-8	sense	AAGGAACCATCTCACTG	352
	antisense	GATTCTTGGATACCACAGAG	
GROα	sense	ATGGCCCGCGCTGCTCTCT	255
	antisense	AGCTTTCCGCCCATTCTTG	
ENA-78	sense	GTGTTGAGAGAGCTGCGTTG	215
	antisense	TTTTCCTTGTTTCCACCGCT	
RANTES	sense	ATGAAGGTCTCCGCGGCACGC	276
	antisense	CTAGCTCATCTCCAAAGAGTT	
MCP-1	sense	TCCTGTGCCTGCTGCTCATAGC	235
	antisense	TTCTGAACCCACTTCTGCTTGG	
cagA	sense	GATAACGCTGTCGCTTCATACG	409
	antisense	CTGCAAAAGATTGTTTGGCAGA	
ureA	sense	GCCAATGGTAAATTAGTT	411
	antisense	CTCCTTAATTGTTTTTAC	

informed consent before their endoscopy. The study was approved by the local clinical research ethics committee.

At endoscopy, biopsy specimens were taken from the antrum and the corpus of the stomach. One biopsy specimen from the antrum and corpus was used for CLO test (Delta West Pty, Australia). Two biopsy specimens from the antrum and the corpus were snap frozen and stored at −80°C before RNA extraction. Two antral and corpus biopsies were taken for histological evaluation. Sections of biopsies were examined without knowledge of the experimental results by one histopathologist (MFD). Mononuclear cell infiltration, neutrophil cell infiltration, and *H pylori* density were graded using a visual analogue scale with units of 0 to 100 for each feature.[15] The modified Giemsa stain was used for identification of *H pylori*.

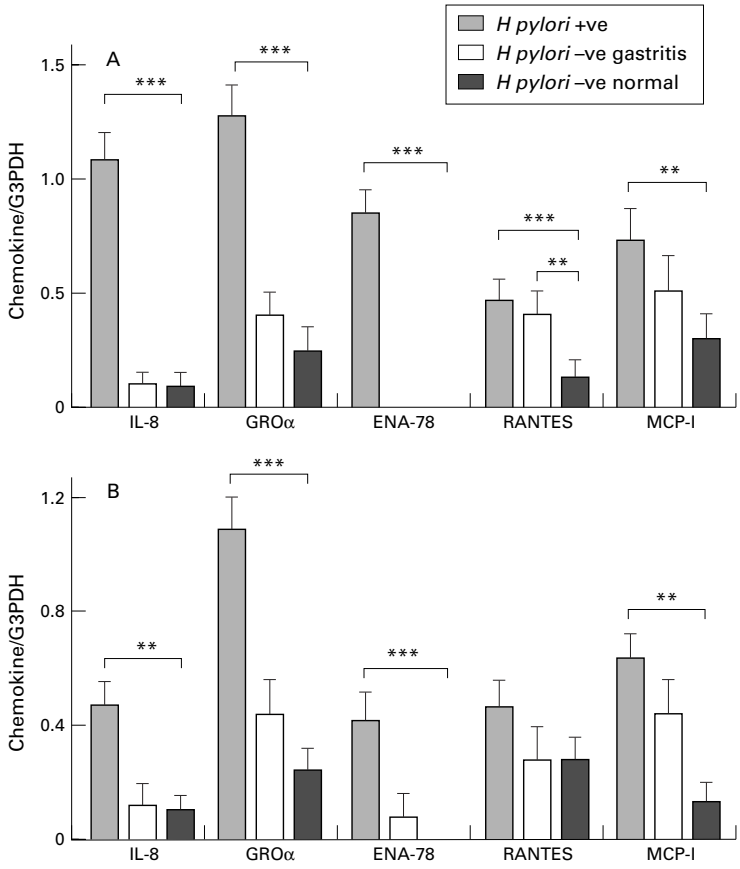

*Figure 1 Chemokine mRNA expression in the antral (A) and corpus (B) mucosa in patients with or without H pylori infection. **p < 0.01, ***p < 0.001 v control group.*

RNA EXTRACTION AND COMPLEMENTARY DNA SYNTHESIS

Total RNA was extracted from biopsy specimens using Catrimox-14™ (Iowa Biotechnology Corporation).[16] Each RNA sample was suspended in water containing 1 U/µl of RNasin™ (ribonuclease inhibitor; Promega). Ten microlitres of each RNA solution was used for reverse transcription using Random Primers™ (random hexamer; Promega). Before reverse transcription, to avoid genomic DNA contamination, RNA samples received deoxyribonuclease treatment using DNase I™ (Gibco BRL). The samples were reverse transcribed in a final volume of 20 µl solution containing 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each dNTPs, 20 U RNasin, 0.5 µg Random Primers, and 120 U M-MLV Reverse Transcriptase™ (Promega). The mixture was incubated at 42°C for 60 minutes and heated at 95°C for 10 minutes.

PCR AND SEMIQUANTITATION OF PCR PRODUCTS

The sequences of primers are shown in table 1. The chemokine and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) primers were designed to include at least one intron to produce different size or no products if any residual genomic DNA was present. One microlitre of cDNA solution was added to 20 µl reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 4.5 mM $MgCl_2$ (ureA) or 3.0 mM $MgCl_2$ (MCP-1) or 1.5 mM $MgCl_2$ (other genes), 200 µM each dNTPs, 20 pmol of each primer, and 1.0 U of Taq DNA polymerase (Promega). After five minutes denaturing at 95°C, 30 cycles (chemokine and G3PDH) or 40 cycles (ureA and cagA) PCR were performed.

PCR products were electrophoresed in 2% agarose gel. Eight microlitre aliquots of each chemokine PCR product were electrophoresed with 8 µl aliquots of G3PDH product in the same well. Quantities of chemokine PCR products were compared with G3PDH products in corresponding mucosa by densitometry.[17] In brief, the gel image was electronically digitised to determine the peak height and the area measurements of each band, and the ratios of chemokines to G3PDH were calculated using a UVP gel documentation system and GelBase software (GDS 5000; Ultra Violet Products).

Products of ureA and cagA were also examined in 2% agarose gel. The sensitivities of ureA and cagA PCR were 50 and 25 colony forming units respectively.

STATISTICAL ANALYSIS

All results of chemokine/G3PDH ratios were expressed as mean (SEM) and were compared

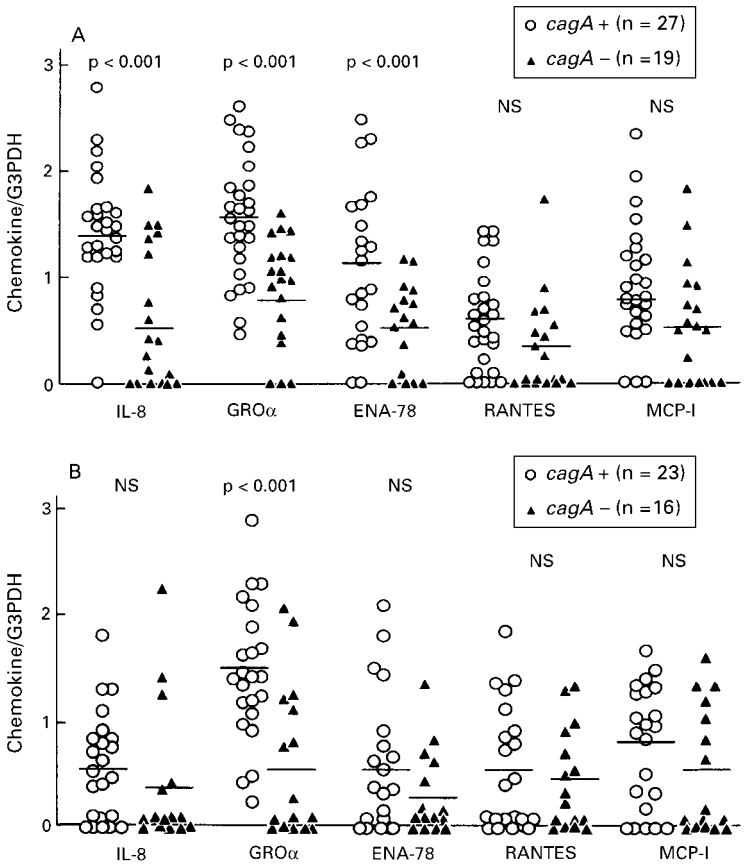

Figure 2 Chemokine mRNA expression in cagA positive and cagA negative antral (A) and corpus (B) mucosa in patients with H pylori infection.

Table 2 Mean chemokine/G3PDH mRNA expression ratio in the antral mucosa in H pylori negative patients

		Gene				
	n	*IL-8*	*GROα*	*ENA-78*	*RANTES*	*MCP-1*
Chronic gastritis	10	0.043	0.577*	0	0.496*	0.465
Reactive gastritis	7	0.247	0.351	0	0.597**	0.816
Normal	20	0.103	0.250	0	0.135	0.299

*p<0.05, **p<0.01 *v* normal.

by the Student *t* test. Determinations of Pearson's correlation coefficient were also made to examine the relation between the chemokine/G3PDH ratios and the visual analogue scales of gastric histopathology. *H pylori* density was expressed as mean (SEM) and the differences were compared by the Student *t* test. A probability (p) value less than 0.05 was considered statistically significant.

Table 3 Correlations between chemokine mRNA expression and mononuclear cell (MNC) infiltration in gastric mucosa with H pylori infection

MNC infiltration	*Gene*				
	IL-8	*GROα*	*ENA-78*	*RANTES*	*MCP-1*
Antrum					
H pylori +	0.389**	0.440**	0.459**	0.203	0.402**
cagA +	0.411*	0.437*	0.416*	0.231	0.391*
cagA−	0.365	0.459*	0.650**	0.101	0.377
Corpus					
H pylori +	0.423**	0.662***	0.712***	0.126	0.171
cagA +	0.428*	0.667***	0.804***	0.017	0.066
cagA−	0.402	0.643**	0.354	0.378	0.266

r Values determined by Pearson correlation coefficient: *p<0.05, **p<0.01, ***p<0.001.

Results

Eighty three patients were included in the study. Endoscopic diagnoses were duodenal ulcer (4), duodenitis (3), gastric ulcer (3), gastric polyp (1), and reflux oesophagitis (5). Antral biopsies were obtained from all patients. The patients were considered *H pylori* positive if at least two of CLO test, histology, and ureA RT-PCR were positive. On this basis 46 patients were defined as *H pylori* positive. Twenty seven of the 46 *H pylori* positive patients were cagA positive. In 37 *H pylori* negative patients, 20 patients had histologically normal mucosa and were defined as the normal control group. In the other 17 *H pylori* negative patients, histologically 10 had chronic gastritis and seven were diagnosed as having reactive gastritis. These patients were classified as the *H pylori* negative gastritis group. There was no difference between groups with respect to age.

Corpus biopsies were obtained for RNA analysis from 63 patients. Thirty nine were *H pylori* positive, and 23 were cagA positive. In the 24 *H pylori* negative patients, histologically 16 patients were classified as the normal control group, three with chronic gastritis (one autoimmune type) and five with reactive gastritis. One patient had *H pylori* negative reactive gastritis in the antral mucosa and cagA positive gastritis in the corpus mucosa.

In cagA positive patients, the mean bacterial density assessed by visual analogue scale was 55.8 (SEM 5.7) units in the antrum and 36.5 (6.2) in the corpus, and the difference was significant ($p < 0.05$). In cagA negative patients, there was no significant difference in the mean bacterial density score in the antrum 39.9 (7.1) and in the corpus 22.5 (5.6). Bacterial density was not significantly different between cagA positive and cagA negative patients in either the antrum or the corpus.

CHEMOKINE mRNA EXPRESSION AND *H PYLORI* STATUS

Chemokine mRNA expression was increased in *H pylori* positive mucosa compared with the normal control group (fig 1). In the antral mucosa, mRNA expression of all chemokines was significantly greater in *H pylori* positive patients than in control patients (fig 1A). In the corpus mucosa, expression of IL-8, GROα, ENA-78, and MCP-1 was also greater in *H pylori* positive patients (fig 1B). In *H pylori* negative patients with gastritis, only RANTES mRNA expression was significantly greater in the antrum than the *H pylori* negative controls (fig 1A).

In the antrum, expression of IL-8, GROα, and ENA-78 mRNA was significantly greater in cagA positive mucosa than in cagA negative mucosa (fig 2A). Although RANTES and MCP-1 mRNA expression was increased in cagA positive mucosa compared with cagA negative mucosa, the differences were not significant (fig 2A). In the corpus mucosa, significant increases in chemokine mRNA expression in cagA positive infection was observed only for GROα (fig 2B).

In 22 cagA positive patients and 16 cagA negative patients, chemokine to G3PDH ratios

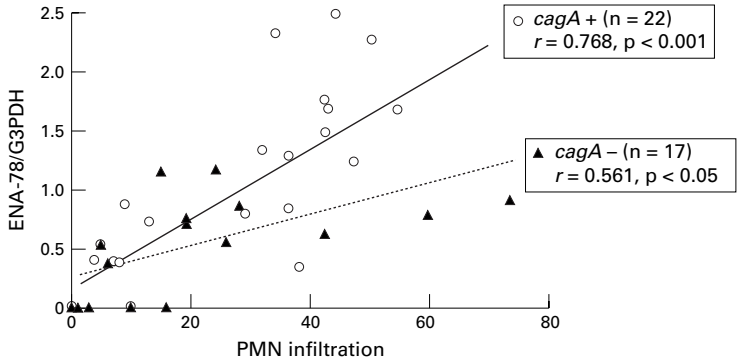

Figure 3 Correlations between neutrophil activity and ENA78 mRNA expression in the antral mucosa in patients with H pylori infection. A solid line and a broken line represent regression lines in cagA positive and cagA negative mucosa respectively.

Table 4 Correlations between chemokine RNA expression and polymorphonuclear neutrophil (PMN) infiltration in gastric mucosa with H pylori infection

PMN infiltration	Gene				
	IL-8	*GROα*	*ENA-78*	*RANTES*	*MCP-1*
Antrum					
H pylori +	0.681***	0.588***	0.656***	0.313	0.190
cagA +	0.691***	0.619***	0.768***	0.256	0.004
cagA –	0.365	0.544*	0.561*	0.309	0.341
Corpus					
H pylori +	0.537***	0.721***	0.828***	0.044	0.159
cagA +	0.517*	0.710***	0.863***	0.036	0.133
cagA –	0.442	0.764***	0.611*	0.244	0.081

r Values determined by Pearson correlation coefficient: *p<0.05, ***p<0.001.

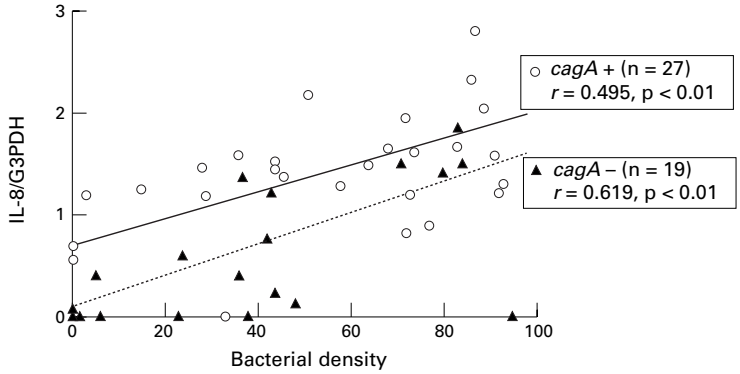

Figure 4 Correlations between bacterial density and IL-8 mRNA expression in the antral mucosa in patients with H pylori infection. A solid line and a broken line represent regression lines in cagA positive and cagA negative mucosa respectively.

Table 5 Correlation between chemokine mRNA expression and bacterial density in gastric mucosa with H pylori infection

Bacterial density	Gene				
	IL-8	*GROα*	*ENA-78*	*RANTES*	*MCP-1*
Antrum					
H pylori +	0.585***	0.585***	0.534***	0.303*	0.346*
cagA +	0.495**	0.701***	0.475*	0.152	0.270
cagA –	0.619**	0.538*	0.664**	0.399	0.331
Corpus					
H pylori +	0.377*	0.425**	0.458**	0.183	0.148
cagA +	0.347	0.288	0.441*	0.064	0.091
cagA –	0.391	0.638**	0.379	0.452	0.146

r Values determined by Pearson correlation coefficient: *p<0.05, **p<0.01, ***p<0.001.

were available both for the antral and the corpus. In cagA positive patients, IL-8 and ENA-78 mRNA expression was significantly greater in the antral mucosa than in the corpus mucosa while there was no significant difference in cagA negative patients. IL-8 to G3PDH ratios for the antrum and corpus were respectively 1.45 (0.12) and 0.52 (0.11), while ENA-78 to G3PDH ratios were 1.12 (0.18) (antrum) and 0.60 (0.16) (corpus). There was no significant difference in expression of GROα, RANTES, and MCP-1 mRNA between the antral and corpus mucosa.

CHEMOKINE mRNA EXPRESSION IN *H PYLORI* NEGATIVE GASTRITIS

The *H pylori* negative gastritis group consisted of patients with both chronic gastritis and reactive gastritis. Subgroup analysis of chemokine mRNA expression in the antral mucosa is shown in table 2. GROα and RANTES mRNA expression was significantly greater in patients with *H pylori* negative chronic gastritis than in the control group. In patients with reactive gastritis patients RANTES mRNA expression was also significantly greater than in the control group.

CORRELATIONS BETWEEN CHEMOKINE mRNA EXPRESSION AND GRADE OF *H PYLORI* GASTRITIS

Significant correlations were found between mononuclear cell infiltration and chemokine mRNA expression (table 3). In cagA positive mucosa, mononuclear cell infiltration was correlated with mRNA expression of IL-8, GROα, ENA-78, and MCP-1 in the antrum, and with IL-8, GROα, and ENA-78 in the corpus. In cagA negative mucosa, significant correlations between mononuclear cell infiltrate and chemokine mRNA expression were found for GROα and ENA-78 in the antrum and GROα in the corpus.

Neutrophil infiltration was significantly correlated with C-X-C chemokine mRNA expression (fig 3, table 4). In cagA positive mucosa, neutrophil infiltration correlated with mRNA expression of IL-8, GROα, and ENA-78 in both the antrum and corpus. In cagA negative mucosa, correlations were significant for GROα and ENA-78 but there was no significant correlation between neutrophil infiltration and IL-8 mRNA expression. No significant correlation was observed between neutrophil infiltration and C-C chemokines.

Bacterial density was also correlated with C-X-C chemokine mRNA expression (fig 4, table 5). In cagA positive mucosa, bacterial density correlated with mRNA expression of IL-8, GROα, and ENA-78 in the antrum, and only with ENA-78 in the corpus. In cagA negative mucosa, correlations were significant with IL-8, GROα, and ENA-78 in the antrum, and only with GROα in the corpus. There was no significant correlation between bacterial density of cagA negative or cagA positive infection and C-C chemokine mRNA expression.

Discussion

There has been recent interest in the role of chemokines in gastric mucosa with *H pylori* infection. In this study, we investigated the mRNA expression of several chemokines in the gastric mucosa using semiquantitative RT-PCR. Our previous in vitro studies with gastric epithelial cells have shown a good correlation between IL-8:G3PDH mRNA using this semiquantitative RT-PCR approach and secreted IL-8 product.[10] The use of visual analogue

scales enabled us to evaluate the correlations between chemokine mRNA expression and histological grade of gastritis. The results suggest that C-X-C chemokines may play a primary role in *H pylori* gastritis.

Both C-X-C and C-C chemokines have been implicated in many chronic inflammatory conditions.[18–24] Gastrointestinal epithelial cells have recently been shown to express and secrete several chemokines.[10–12 25] Chemokine expression is induced in both human colonic epithelial cells and mononuclear cells in response to bacterial infection.[26 27] Increased colonic C-X-C and C-C chemokines are found in inflammatory bowel disease.[28 29] Although early studies showed increased IL-8 production in gastric mucosa infected with *H pylori*,[5 6] the involvement of other chemokines in gastritis has not been investigated in detail. Our results show that patients infected with *H pylori* have significantly greater mRNA expression of both C-X-C and C-C chemokines in the gastric mucosa than patients without *H pylori* infection.

Many studies have focused on strain diversity of *H pylori* and they have shown that infection with cagA positive strains is closely associated with gastroduodenal diseases.[30–37] Infection with cagA positive strains is associated with increased gastric IL-8 mRNA expression[8] and IL-8 protein in vivo.[9] In vitro studies have also shown that the ability of *H pylori* to induce chemokines in gastric epithelial cell lines varies and the response is restricted to strains with the cagA phenotype.[10–14 38] Our present study shows that patients infected with cagA positive strains have greater gastric C-X-C chemokine mRNA expression than those infected with cagA negative *H pylori*. Atherton *et al* showed that cagA positive strains colonise at a greater density than cagA negative strains.[39] However, others have found no significant difference in colony forming units between cagA positive and cagA negative patients.[40] In the present study, although the bacterial densities were higher in cagA positive patients, the difference was not significant. Thus cagA positive strains appear to induce greater C-X-C chemokine mRNA expression in the gastric mucosa. This is apparent particularly at low bacterial density (fig 4).

Interestingly, expression of IL-8 and ENA-78 mRNA was greater in the antrum than in the corpus only in patients with cagA positive infection. Variations in bacterial density could be one explanation for differences in chemokine mRNA expression between antrum and corpus. In cagA positive patients, bacterial density was significantly higher in the antrum than in the corpus ($p < 0.05$). By contrast, no difference was seen in cagA negative patients. High acid in the corpus mucosa may also affect bacterial–epithelial interactions by influencing transcription of bacterial genes in the cag PAI and other related virulence genes.[41] Differences in chemokine induction could explain why inflammation in cagA seropositive patients is significantly increased relative to cagA seronegatives only in the antral mucosa.[42 43] Furthermore, Bayerdorffer *et al* showed *H pylori* infection of similar density caused a higher grade of gastritis in the antrum than in the corpus.[44] Their findings and our results suggest that increased chemokine stimulation may contribute to enhanced immunological responses to *H pylori* in the antrum rather than in the corpus.

In this study, the grade of gastritis was evaluated using visual analogue scales, which allows more precise examination of the correlations between histology and chemokine mRNA expression. Previous studies have shown increased IL-8 protein and mRNA expression in gastric mucosa is associated with greater mononuclear cell infiltration or neutrophil infiltration, as classified by the Sydney system.[6 8 9 45] The correlations between IL-8 mRNA expression and mononuclear cell infiltration and neutrophil activity were significant in *H pylori* positive gastric mucosa. We also examined these correlations in cagA positive and cagA negative infection and showed significant correlations for IL-8 and neutrophil infiltration only in patients infected with cagA positive strains. The mRNA expression of other C-X-C chemokines, GROα, and ENA-78 was also positively correlated with both mononuclear cell and neutrophil infiltration in *H pylori* positive gastric mucosa. As expected, correlations of C-X-C chemokine mRNA expression were stronger with neutrophil infiltration than with mononuclear cell infiltration. C-X-C chemokines are likely to play an important role in gastric mucosal neutrophil migration and account for the more active gastritis associated with cagA positive infection. The correlations between C-C chemokine mRNA expression and mononuclear cell and neutrophil infiltration were quite different. Significant correlation was seen only between MCP-1 mRNA expression and mononuclear cell infiltration. Though C-C chemokine mRNA expression was increased in *H pylori* positive gastric mucosa, its importance might not be comparable to that of the C-X-C chemokines. Additionally, in patients with *H pylori* negative gastritis who had some mononuclear cell infiltration but no neutrophil infiltration, the mRNA expression of the C-C chemokine RANTES is increased.

We also examined the correlations between bacterial density and chemokine mRNA expression. Bacterial density was correlated with mRNA expression of all chemokines in *H pylori* positive antral mucosa. However, these correlations were generally stronger in cagA negative patients than in cagA positive patients. Greater chemokine mRNA expression in cagA positive infection at low bacterial densities will be a result of induction of epithelial chemokines by cag PAI genes. In contrast, induction of chemokines in cagA negative infection will depend on mucosal mononuclear cell activation and relate more strongly to bacterial density. Recent studies have shown that IL-8 protein content of antral biopsies is related to the density of *H pylori* infection.[45]

In conclusion, chemokines have a role in *H pylori* positive gastritis. Enhanced C-X-C chemokine responses are induced by cagA

positive *H pylori* strains resulting in histologically more intense gastritis. Infection with cagA positive strains is associated clinically with more severe outcomes, and variability in host chemokine responses may thus contribute to the clinical outcome of *H pylori* infection.

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