Increased expression of IL-10 and IL-12 (p40) mRNA in *Helicobacter pylori* infected gastric mucosa: relation to bacterial cag status and peptic ulceration

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Abstract

***Aims*—To investigate interleukin (IL)-12 (p40) and IL-10 mRNA expression levels in the gastric mucosa in relation to *H pylori* cag status, peptic ulceration, and histopathology.**

***Methods*—In 81 dyspeptic patients, antral and corpus biopsies were taken for reverse transcriptase polymerase chain reaction (RT-PCR) and histology. G3PDH (control) and IL-10 and IL-12 were co-amplified in a duplex PCR and the ratios of cytokines to G3PDH were determined. Bacterial ureA and cagA status was determined by RT-PCR.**

***Results*—IL-10 mRNA expression in both the antral and corpus mucosa was greater ($p < 0.01$) in cagA positive infection than in *H pylori* negative patients with histologically normal mucosa. No increase in IL-10 mRNA expression was observed in cagA negative infection. Both in the antral and corpus mucosa, IL-12 mRNA expression was greater ($p < 0.05$) in cagA positive than in cagA negative infection and uninfected patients with normal gastric mucosa. In cagA positive infection, there was a correlation between IL-10 and IL-12 mRNA expression in both the antral mucosa ($r = 0.515$, $p < 0.01$) and the corpus mucosa ($r = 0.6$, $p < 0.005$). IL-12 mRNA expression in the antral mucosa was significantly more frequent in *H pylori* positive patients with duodenal ulcer than in those with gastric ulcer or non-ulcer dyspepsia. No difference was observed in IL-10 mRNA expression in relation to endoscopic diagnosis.**

***Conclusions*—CagA positive *H pylori* infection is associated with increased IL-10 and IL-12 mRNA expression. The increased expression of IL-12 mRNA in the majority of patients with duodenal ulcer suggests that Th1 responses may predominate and play a role in the pathogenesis of duodenal ulceration.**

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Keywords: *H pylori*; cag pathogenicity island; IL-10; IL-12; peptic ulcer

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In *Helicobacter pylori* infection, the inflammatory immune response generated by the bacterium is likely to be a major factor contributing to gastric mucosal damage.[1] Cytokines play a critical role in the regulation of initial acute inflammation and specific T and B cell responses induced by *H pylori*. Recent studies in the *H felis* mouse model suggest an important role of specific T cell responses in the induction of gastric mucosal inflammation.[2 3] Both in mice and humans, CD4+ T helper (Th) cells can be divided into two main subsets, based on their differential cytokine production profiles.[4 5] Human Th1 cells, which promote cell mediated immune responses, produce high levels of interferon γ (IFNγ) but no IL-4 and IL-5, whereas Th2 cells, which induce humoral responses, produce IL-4 and IL-5 but no IFNγ.[4] Cytokines, such as interleukin (IL)-12 and IL-10, are known to influence the differentiation of T helper cells.

IL-12 is a heterodimeric cytokine composed of two unrelated chains, p40 and p35, encoded by separate genes located on different chromosomes.[6] The expression of the p40 gene is specific to IL-12 producing cells, while the p35 gene expression is constitutively expressed in different cell types. The production of both chains is required to form a biologically active heterodimer (p70).[7] IL-12 is produced mostly by phagocytic cells in response to bacterial infection. Together with IFNγ, IL-12 induces the differentiation of Th1 cells and inhibits Th2 responses.[6]

IL-10 was initially described as a product of Th2 cells which inhibits the secretion of cytokines by Th1 cells.[8] However, recent studies have shown that human IL-10 is not strictly a Th2 specific cytokine.[9] IL-10 inhibits the differentiation of Th1 cells by suppressing IL-12 production from accessory cells.[6 9 10] IL-10 also has anti-inflammatory properties, inhibiting the production of proinflammatory cytokines and chemokines from macrophages and neutrophils.[11]

Current evidence from both human[12–15] and murine studies[2 3] has shown that Th1 responses predominate during chronic helicobacter associated gastritis. A recent study on *H pylori* specific gastric T cell clones suggests that the mucosal T cell response to *H pylori* in patients with peptic ulceration is more polarised towards a Th1 profile than in those with chronic gastritis only.[12] Only 11 subjects were studied and all were infected with cytotoxin associated gene A (cagA) positive strains.[12] In vivo infection with *H pylori* strains expressing cagA, which is part of the cag pathogenicity island (PAI), is associated with enhanced

Table 1 Oligonucleotide primers for reverse transcriptase polymerase chain reaction

Gene		Primer	Expected product size (bp)
G3PDH	Sense	GAGTCAACGGATTTGGTCGT	158
	Antisense	GGTGCCATGGAATTTGCCAT	
IL-10	Sense	AGTCGCCACCCTGATGTCTC	223
	Antisense	CCTGGGGGAGAACCTGAAG	
IL-12 (p40)	Sense	CCTGCTGGTGGCTGACGACAAT	311
	Antisense	CTTCAGCTGCAAGTTGTTGGGT	
ureA	Sense	GCCAATGGTAAATTAGTT	411
	Antisense	CTCCTTAATTGTTTTTAC	
cagA	Sense	GATAACGCTGTCGCTTCATACG	409
	Antisense	CTGCAAAAGATTGTTTGGCAGA	

bp, base pair; IL, interleukin.

chemokine responses,[16–20] more severe gastric inflammation and increased risk of peptic ulceration, gastric atrophy, and gastric cancer of the intestinal type.[21–23]

Recent studies on gastric mucosal expression of IL-10[16 24–28] and IL-12[13 26 27] in *H pylori* associated gastritis have given varying results. This may reflect both methodology and bacterial phenotype. No studies to date have fully characterised gastric mRNA expression of IL-12 in relation to cag status or peptic ulceration. In contrast to earlier studies,[16 25] we have used semiquantitative techniques[19] to investigate IL-12 (p40) and IL-10 mRNA expression levels in the gastric mucosa in relation to bacterial cag status, peptic ulceration, and histopathology.

Methods

PATIENTS

We studied 81 patients with dyspeptic symptoms (36 male and 45 female, age range 17 to 69 years, mean age 45.4 years). Patients who had received antisecretory agents, antibiotics, bismuth, or non-steroidal anti-inflammatory drugs (NSAID) within the previous two months were excluded. Patients who had received *H pylori* eradication treatment were also excluded. Informed consent was obtained from all patients and the study was approved by the local clinical research ethics committee.

SAMPLE COLLECTION AND HISTOPATHOLOGY

During upper gastrointestinal endoscopy, multiple biopsy specimens were taken from the gastric antrum and corpus. One biopsy specimen from the antrum was used for the rapid urease test (CLO test, Delta West Pty, Australia). Two biopsy specimens from the antrum and corpus were snap frozen in liquid nitrogen and stored at −80°C before RNA extraction. Two antral and corpus biopsies were taken for histological examination, including modified Giemsa staining for the identification of *H pylori*. Specimens were examined without knowledge of the experimental results by one histopathologist (MFD). Chronic inflammation, polymorphonuclear activity, atrophy, intestinal metaplasia, and *H pylori* colonisation density were graded from 0 to 3 according to the updated Sydney system.[29] The degree of antral predominance for these features was assessed by subtracting corpus scores from antral scores for each patient.[30]

RNA EXTRACTION AND REVERSE TRANSCRIPTION

Total RNA was extracted from biopsy specimens using a cationic detergent based extraction method (Catrimox-14, Iowa Biotechnology), following the manufacturer's protocol.[31] Isolated RNA was dissolved in 20 µl of RNA solubilisation solution containing 20 U of ribonuclease inhibitor (RNasin, Promega) and 20 mM dithiothreitol. To avoid genomic DNA contamination, RNA samples received deoxyribonuclease treatment using 1 U of DNase ITM (Gibco BRL) before reverse transcription. Ten microlitres of each RNA sample were reverse transcribed with 0.5 µg random hexamer (Random Primers, Promega), 120 U Moloney murine leukaemia virus reverse transcriptase (MMLV-RT, Promega), 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 1.0 mM each dNTPs, and 20 U RNasin, in a final volume of 20 µl. The mixture was incubated at 42°C for one hour, then heated to 95°C for five minutes and stored at 4°C until use.

PCR AND SEMIQUANTITATION OF PCR PRODUCTS

The sequences of the oligonucleotide primer pairs used in this study are shown in table 1. glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and each of IL-10[32] and IL-12 (p40)[26] were coamplified in a duplex polymerase chain reaction (PCR). One microlitre of complementary DNA was added to the PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4.5 mM (ureA) or 2.0 mM (IL-10, IL-12, and G3PDH) or 1.5 mM (cagA) $MgCl_2$, 200 µM each dNTPs, primer pairs (combined at a ratio of 1:20 pmol for G3PDH to IL-10 and 1:30 pmol for G3PDH to IL-12), and 1.0 U Taq DNA polymerase (Promega).

Amplification was performed in a thermal cycler: five minutes at 95°C (initial denaturation) followed by 35 cycles (IL-10, IL-12, and G3PDH) or 40 cycles (ureA and cagA) of one minute denaturation at 95°C, one minute annealing at 50°C (ureA and cagA) or 55°C (IL-10 and G3PDH) or 60°C (IL-12 and G3PDH), and one minute extension at 72°C. The final cycle included extension for five minutes at 72°C. Negative and positive control amplifications were performed in each PCR series.

Randomly selected bacterial RNA was used for ureA and cagA PCR to check for bacterial genomic DNA contamination and no product was amplified. Products of PCR were electrophoresed on 2% agarose gel and visualised by ethidium bromide staining under ultraviolet light. The gel was run with a track containing loading buffer and negative control to act as a background track for subtraction during image analysis. The image was electronically captured and digitised using a UVP gel documentation system (GDS 5000; Ultra Violet Products). The peak height and the area measurements were determined for each band on the track using Gelbase software and the ratios of cytokines to G3PDH were calculated.[33]

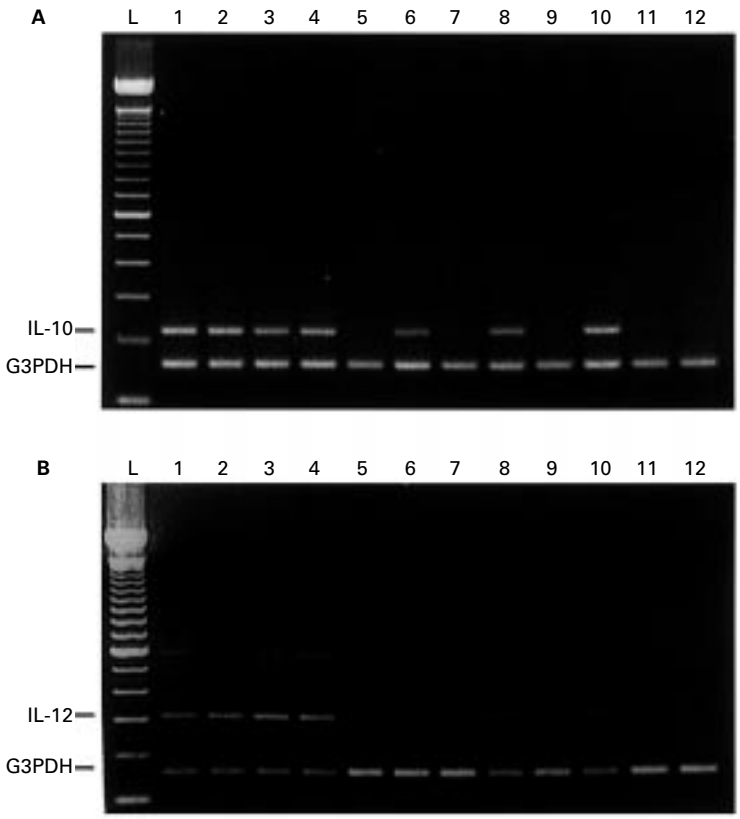

Figure 1 Representative reverse transcriptase polymerase chain reaction (RT-PCR) for (A) IL-10 and (B) IL-12 (p40) mRNA in gastric antral biopsies. Lane L, 100 base pair ladder; lanes 1–4, H pylori ureB positive, cagA positive gastritis; lanes 5–8, H pylori ureA positive, cagA negative gastritis; lanes 9 and 10, H pylori negative gastritis; lanes 11 and 12, H pylori negative normal mucosa.

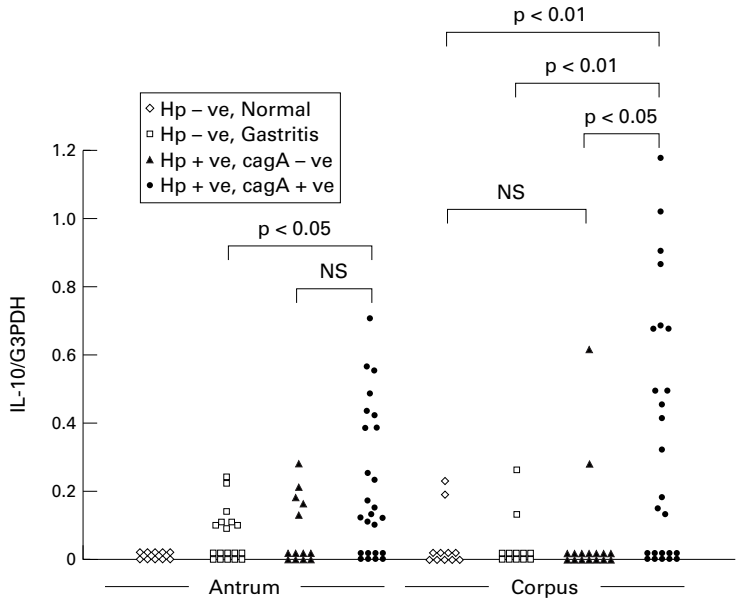

Figure 2 Expression of interleukin (IL)-10 mRNA in the gastric antral and corpus mucosa in H pylori negative and positive patients.

STATISTICAL ANALYSIS

The χ^2 test with Yates' correction was used to compare the frequencies of cytokine mRNA expression in different patient groups. Results of the ratios of cytokines to G3PDH in different groups were compared by the Mann–Whitney U test. The relation between the IL-10 to G3PDH and IL-12 to G3PDH ratios and histological features was determined by the Pearson correlation coefficient. A p value of less than 0.05 was considered statistically significant.

Results

H PYLORI STATUS, HISTOLOGICAL FINDINGS, AND ENDOSCOPIC DIAGNOSIS

The patients were defined as *H pylori* positive if at least one of CLO test, histological examination, or ureA reverse transcriptase polymerase chain reaction (RT-PCR) was positive. Forty seven of 81 patients (58%) were *H pylori* positive and 30 of these (64%) were cagA positive by RT-PCR. Histologically, the antral biopsies of all *H pylori* positive patients showed chronic active gastritis. In 34 *H pylori* negative patients, 15 had histologically normal gastric mucosa and were classified as the normal control group. The other 19 *H pylori* negative patients with some form of gastritis histologically were classified as the *H pylori* negative gastritis group. This group consisted of 10 patients with chemical or reactive gastritis, seven with inactive chronic gastritis, and two with autoimmune type atrophic gastritis. Endoscopic findings in the patients studied were as follows: 12 patients (age range 27 to 68 years, mean age 48.8 years) had active duodenal ulcer or scar; five patients (age range 43 to 62, mean age 54.4) had gastric ulcer; and 64 patients (age range 17 to 69, mean age 47.6) had no endoscopic evidence of ulceration. Thirty of 64 patients (47%) without endoscopic ulcers and all of those with duodenal and gastric ulcers were *H pylori* positive. Positivity for cagA gene did not differ in the three groups (duodenal ulcer, 67%; gastric ulcer, 60%; non-ulcer group, 63%).

IL-10 AND IL-12 (p40) mRNA EXPRESSION AND *H PYLORI* INFECTION

In the 81 patients, antral biopsies were obtained from 66 and corpus biopsies from 64 for RT-PCR. Representative results of RT-PCR for IL-10 and IL-12 (p40) are shown in fig 1.

In the antral (A) and corpus (C) mucosa, positivity for IL-10 mRNA expression was significantly more frequent ($p < 0.05$) in *H pylori* positive patients (A, 58%; C, 42%) than in *H pylori* negative patients (A, 29%; C, 17%). IL-10 mRNA expression (IL-10 to G3PDH ratio) in both antral and corpus mucosa was higher ($p < 0.01$) in cagA positive infection than in normal control patients. IL-10 mRNA expression in cagA negative infection was not significantly different from uninfected control patients in either the antral or corpus mucosa (fig 2). Corpus IL-10 mRNA expression was greater ($p < 0.05$) in cagA positive than in cagA negative infection (fig 2). In *H pylori* negative patients with chronic gastritis, IL-10 mRNA expression was not significantly different from the *H pylori* negative control group in either the antral or the corpus mucosa (fig 2).

IL-12 (p40) mRNA positivity was more frequent in *H pylori* positive patients (A, 29%; C, 34%, $p < 0.05$) than in *H pylori* negative

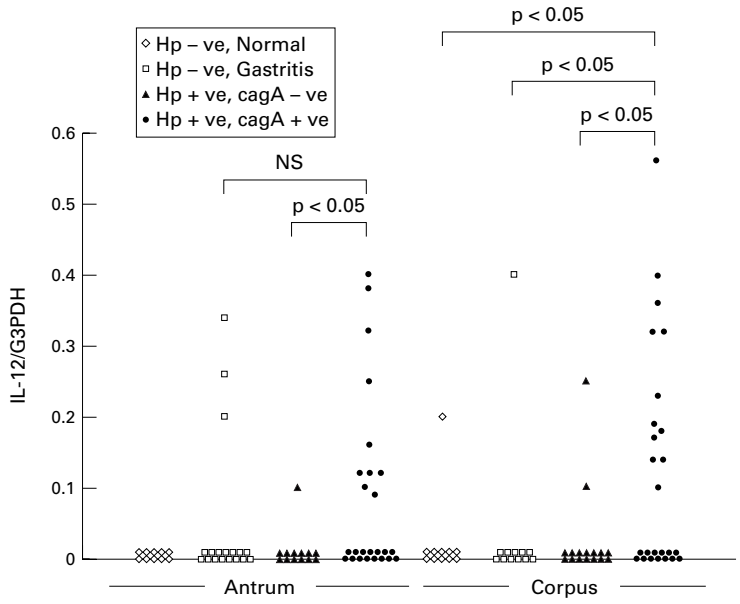

Figure 3 Expression of interleukin (IL)-12 (p40) mRNA in the gastric antral and corpus mucosa in H pylori negative and positive patients.

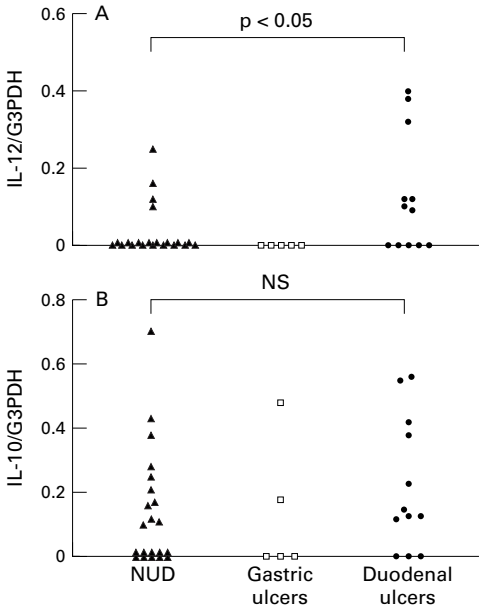

Figure 4 Expression of antral interleukin (IL)-12 (p40) mRNA (A) and IL-10 mRNA (B) in H pylori positive patients without ulceration (NUD) and with duodenal ulcers or gastric ulcers (as the ratio of IL-10 or IL-12 to glyceraldehyde 3-phosphate dehydrogenase).

patients (A, 11%; C, 9%). IL-12 mRNA expression (IL-12 to G3PDH ratio) in both antral and corpus mucosa was greater ($p < 0.05$) in cagA positive infection than in *H pylori* negative normal control patients with histologically normal mucosa (fig 3). Both in the antral and corpus mucosa, IL-12 mRNA expression was greater ($p < 0.05$) in cagA positive than in cagA negative infection. IL-12 mRNA expression in cagA negative infection was no different from that in uninfected patients with normal gastric mucosa. In cagA positive infection, there was a significant correlation between IL-10 and IL-12 mRNA expression in both the antral mucosa ($r = 0.515$, $p < 0.01$) and the corpus mucosa ($r = 0.6$, $p < 0.005$). This correlation was not observed in cagA negative infection. Among the *H pylori* positive patients, no correlation was observed between IL-10 and IL-12 mRNA expression and chronic inflammation, polymorphonuclear cell activity, atrophy, intestinal metaplasia, and *H pylori* colonisation density. Furthermore, there was no correlation between the degree of antrum predominant gastritis and mRNA expression of either cytokine. In *H pylori* negative patients with chronic gastritis, the IL-12 to G3PDH ratios did not differ from the *H pylori* negative control group (fig 3).

IL-10 AND IL-12 (p40) mRNA EXPRESSION AND ENDOSCOPIC DIAGNOSIS

In *H pylori* infected patients, antral IL-12 mRNA expression was greater ($p < 0.05$) in those with duodenal ulcer than in those without ulcers (fig 4A). In *H pylori* positive patients with duodenal ulcer, six of seven patients with increased IL-12 mRNA expression in the antral mucosa were cagA positive. Interestingly, in *H pylori* positive patients without ulcers, all patients with increased levels of IL-12 mRNA in the antral mucosa were also cagA positive. Positivity for IL-12 mRNA in the antral mucosa was found more often ($p < 0.05$) in patients with duodenal ulcer (58%) than in those with gastric ulcer (0%). IL-12 mRNA expression in the corpus mucosa was also more common in patients with duodenal ulcer (50%) than in those with gastric ulcer (25%) or without ulcers (30%). The observed variation in IL-12 mRNA expression between patients with duodenal ulcers and gastric ulcers did not relate to any significant differences in gastric histopathology between the two groups. No difference was observed in IL-10 mRNA expression in relation to endoscopic diagnosis in either the antral mucosa (fig 4B) or the corpus mucosa.

Discussion

Current evidence from both human[12–15] and murine studies[2 3] has shown that the T helper response in the gastric mucosa with chronic helicobacter infection has a predominantly Th1 phenotype, characterised by high IFNγ and low IL-4 production. IL-12, which is produced by phagocytic cells, antigen presenting cells, and B cells in response to bacteria and bacterial products, plays an important role in the differentiation of Th1 cells.[6 34–36] IL-12 deficient mice are defective in their ability to secrete IFNγ in response to several antigens and to generate normal Th1 responses.[37]

In *H pylori* gastritis, variable results have been reported on mucosal IL-12 mRNA expression. Karttunen *et al*[26] and D'Elios *et al*[12] observed increased gastric IL-12 mRNA expression in *H pylori* infection; however, in another study no difference in IL-12 mRNA expression or biopsy IL-12 protein in *H pylori* infection was found.[27] In our study, IL-12 mRNA expression was increased in cagA positive *H pylori* infected antral and corpus mucosa, but not in cagA negative infection, possi-

bly accounting for earlier discrepant observations. Increased IL-12 in cagA positive infection may promote strong Th1 cell mediated responses, which are considered to be associated with increased mucosal damage.[1] The important role of Th1 responses in mucosal damage has recently been demonstrated in the *H felis* infected mouse model.[2 3] Neutralisation of IFNγ reduced the severity of gastric inflammation[2] and passive transfer of Th1 cell lines exacerbated gastritis.[3]

Recently, D'Elios *et al* suggested that gastric Th1 responses are more frequent in patients with peptic ulcer disease than in those with chronic gastritis only.[12] Their study investigated the cytokine profile of *H pylori* specific T cell clones generated from cagA positive *H pylori* infected gastric biopsies. The majority of *H pylori*-specific T cell clones from the antral mucosa of six patients with peptic ulceration showed a Th1 profile secreting both IFNγ and tumour necrosis factor α (TNFα). In contrast, 64% of clones from five non-ulcer patients with chronic gastritis expressed a Th0 profile, secreting both Th1 and Th2 cytokines following exposure to *H pylori* antigens.[12] In our study, antral IL-12 mRNA expression was increased in the majority of *H pylori* infected patients with duodenal ulcers but not in those with gastric ulcers or without ulcers. This result suggests that in patients with duodenal ulcer, mucosal Th1 responses may predominate and be a factor in the pathogenesis of duodenal ulceration.

In many populations the presence of cagA positive *H pylori* strains has been linked to increased risk of peptic ulceration.[21 25 38 39] The frequency of cagA positive strains globally is highly variable[40] and in some populations with a high frequency of cagA positive strains an association with ulceration has not been observed.[41] In our study, positivity for gastric IL-12 mRNA was observed in approximately half of those infected with cagA positive strains. It will be interesting to examine gastric IL-12 mRNA expression, the cagA status of *H pylori*, and peptic ulceration in other populations.

Th1 responses may contribute to mucosal damage directly by causing cytotoxic damage to epithelial cells or by changing epithelial phenotype, with increased expression of HLA class II molecules.[42] This phenotypic change, and the changes in epithelial permeability that will be mediated by IFNγ and TNFα,[43 44] may facilitate enhanced antigen presentation by both epithelial cells and intramucosal antigen presenting cells causing an exacerbation of mucosal inflammation. The predominate Th1 responses in patients with duodenal ulcer may also be relevant to the perturbations in gastric physiological responses associated with ulceration.[45] A recent in vitro study showed that IFNγ stimulates gastrin secretion from canine antral G cells.[46]

Anti-inflammatory cytokines such as IL-10 are important in the downregulation of excessive proinflammatory responses[10] and inhibit Th1 differentiation by suppressing IL-12 secretion from accessory cells.[6 9 11] Recently, Groux *et al* reported that IL-10 stimulates the generation of a T cell subset, designated a T regulatory cell 1 (Tr1), which produces high levels of IL-10 and has immunoregulatory properties.[47] Tr1 clones can prevent T cell mediated colitis in mice with severe combined immune deficiency,[47] showing the importance of IL-10 for the maintenance of T cell tolerance in the gastrointestinal mucosa. IL-10 mRNA expression is increased in human intestinal mucosal cells in inflammatory bowel disease.[48] In patients with inflammatory bowel disease, particularly Crohn's disease, chronic intestinal inflammation is characterised by Th1 predominant responses.[4 48 49] IL-10 may downregulate the increased secretion of proinflammatory cytokines in such patients,[50] and a similar role is feasible in helicobacter associated gastritis. Recently, Berg *et al* reported that *H felis* infected IL-10 deficient, but not wild type, mice develop a severe hyperplastic gastritis with marked epithelial proliferation and dedifferentiation.[51] This result strongly suggests that IL-10 may be a key factor in the host's immune responses to gastric helicobacter infection.

There have been variable reports on gastric expression of IL-10 mRNA[16 25–27] and protein[25 27 28] in *H pylori* infection. Some studies[26] have not assessed IL-10 mRNA directly in snap frozen biopsies as in this study, but have examined mRNA expression in enzymatically extracted gastric cells. The cell isolation procedures are likely to induce cytokine expression. Measurement of cytokines in biopsy homogenates[27 28] is also likely to be complicated not only by the sensitivity of the cytokine ELISA, but also by the presence of mucosal autoantibodies to cytokines[52] and cytokine receptors.[53] In agreement with two earlier studies,[16 25] we observed increased IL-10 mRNA expression in *H pylori* infected gastric mucosa. Increased IL-10 mRNA was seen predominantly in patents with cagA positive infection; thus differences in cag status could account for earlier variable results. Several reports have shown that infection with cagA positive strains is associated with increased gastric C-X-C chemokine expression and severe gastric inflammation.[16–20] IL-10, which inhibits the secretion of chemokines from macrophages and polymorphonuclear cells,[54] may be an important defence mechanism protecting against enhanced C-X-C responses in cagA positive infection.

Interestingly recent studies have shown that IL-12 can induce T cells to secrete IL-10.[55] Our finding of a significant correlation between IL-10 and IL-12 mRNA expression in cagA positive *H pylori* infection suggests that IL-12 may limit its own production by induction of IL-10 as a negative feedback for IL-12 induced Th1 responses. In our study, no difference was observed in IL-10 mRNA expression in *H pylori* infected patients in relation to peptic ulceration. This suggests that lack of IL-10 is unlikely to be a contributory factor in peptic ulceration.

CONCLUSIONS

Expression of both IL-10 and IL-12 mRNA is increased in cagA positive *H pylori* infection. Increased expression of IL-12 mRNA in cagA positive infection may polarise the differentiation of naive T cells into Th1 cells and promote cell mediated responses. In patients with duodenal ulcer, IL-12 regulated mucosal Th1 responses may predominate and play a role in the pathogenesis of duodenal ulceration. IL-10, which inhibits the secretion of pro-inflammatory cytokines and chemokines, may be an important defence mechanism protecting against the exaggerated C-X-C and Th1 responses in cagA positive infection. The balance between induction of proinflammatory cytokines and stimulation of anti-inflammatory cytokines may be important in disease outcome.

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