Cytokine gene expression in *Helicobacter pylori* associated antral gastritis

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Abstract

Infection of the gastric antrum by *Helicobacter pylori* is characterised by a cellular inflammatory infiltrate. Whether cytokines are involved in the pathogenesis of this gastritis has been investigated by studying the effect of eradicating *H pylori* on the expression of genes encoding the cytokines interleukin 8 (IL-8) and tumour necrosis factor α (TNF-α) in the antral mucosa. Gastric antral biopsy specimens were taken from nine patients with duodenal ulcers and cytokine transcripts were identified and quantified by northern blotting. After *H pylori* had been eradicated the chronic inflammatory infiltrate decreased in all the patients and the polymorphonuclear infiltrate virtually disappeared. Expression of genes also decreased. After eradication, the median TNF-α mRNA/rRNA fell to 48% (p=0·02) and the median IL-8 mRNA/rRNA fell to 5% (p=0·004) of initial values. These results support the role of increased synthesis of these cytokines in the pathogenesis of the gastritis.

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Patients with duodenal ulcer disease almost always have *Helicobacter pylori* infection of the gastric antrum and an associated antral gastritis, comprising a mucosal infiltrate of polymorphonuclear and mononuclear leukocytes. Eradication of *H pylori* from these individuals leads to a resolution of the gastritis and a sustained remission from duodenal ulcer disease.[1]

It is not currently clear how the antral gastritis is caused. In vitro, *H pylori* secretes several factors that may contribute to the inflammatory process. These include potent neutrophil chemotaxins,[2–4] and substances capable of activating peripheral blood monocytes to produce the cytokines interleukin 1β (IL-1β) and tumour necrosis factor α (TNF-α).[5] In addition, a number of cytokines have been identified in the supernatants of *H pylori* infected gastric mucosa in short term organ culture. Crabtree *et al* were able to detect interleukin 8 (IL-8) release from 84% of samples of histologically normal mucosa, but found significantly greater release from infected mucosa.[6] Preliminary reports from other groups confirm increased release of IL-8.[7 8] Crabtree *et al* previously demonstrated an increased release of other cytokines, IL-6[9] and TNF-α[9] from mucosa infected with *H pylori*.

The secretion of potent proinflammatory molecules such as TNF-α and IL-8 leads to tissue damage by causing the recruitment and activation of the host's leukocytes. The finding of raised concentrations of a cytokine secreted into organ culture medium and increased tissue levels suggest increased release in vivo. To examine further the evidence implicating cytokines in the pathogenesis of *H pylori* associated antral gastritis in vivo we attempted to measure the antral mucosal concentrations of their respective mRNAs, an index of their rate of synthesis.

In this report we describe how we have identified the mRNAs encoding TNF-α and IL-8 in the antral mucosa of duodenal ulcer patients by northern blotting. Using this method we have found that eradication of *H pylori* with associated improvement of the gastritis led to a reduction in the mRNAs encoding both of these cytokines.

Methods

PATIENTS AND BIOPSIES

The study was approved by the Hammersmith Hospital Research Ethics Committee. Nine patients with endoscopically proved duodenal ulcers associated with *H pylori* infection were recruited from the gastroenterology outpatient clinic and informed consent was obtained. The patients were aged 20–71 years (mean 51) and four were men. All the patients were in good general health. None had a history of gastric surgery, had taken antibiotics or bismuth preparations in the preceding six months or histamine-H_2 receptor antagonists, omeprazole, or non-steroidal anti-inflammatory drugs during the 14 days before their initial endoscopy.

At the time of the initial endoscopy, four gastric antral biopsy specimens were taken with Olympus biopsy forceps, cup size 5×2 mm (Key-Med, Southend on Sea, Essex) for the diagnosis of *H pylori*. One specimen was used for a urease test,[10] two for histology, and one for bacterial culture. A further five antral biopsy specimens per patient were snap frozen together in liquid nitrogen for RNA extraction.[11] The patients were then treated with tripotassium dicitratobismuthate (DeNol; 120 mg four times daily for one month), with metronidazole (400 mg three times daily), and tetracycline (500 mg four times daily) taken concurrently for the first two weeks. Four weeks after the end of treatment the patients were endoscoped again and biopsy specimens taken as before. Successful eradication of *H pylori* was presumed to have occurred if all the tests were negative at the follow up endoscopy.

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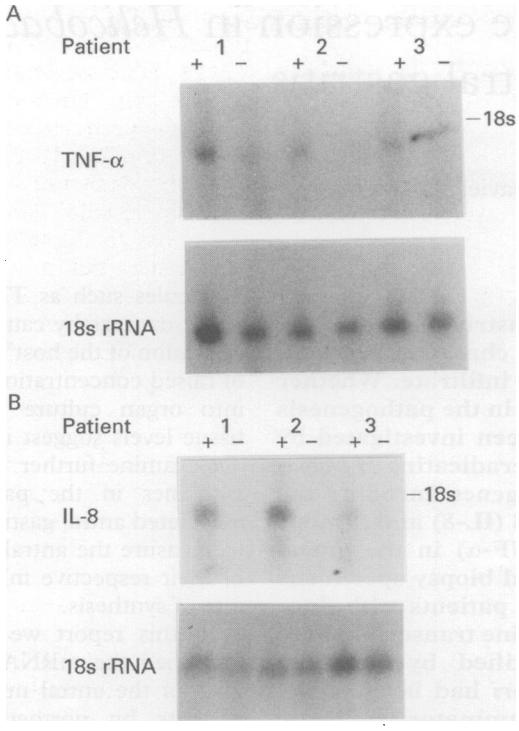

Figure 1: Autoradiographs of northern hybridisations of RNA from three patients, before (+) and after (−) the eradication of Helicobacter pylori, *with probes for (A) TNF-α and (B) IL-8. Ribosomal 18s RNA was probed to control for uneven loading. The position that it migrated to is indicated on the right.*

MEASUREMENT OF mRNA

Total RNA was extracted and 10 μg samples were run on 1% agarose formaldehyde gels and fixed into nylon membranes as described previously.[11] To minimise any variability in the transfer of RNA, paired samples from the same patient – taken before and after triple therapy – were run on adjacent gel lanes.

The membranes were then hybridised with ^{32}P-labelled cDNA probes for either IL-8 or TNF-α, stripped, and reprobed with an oligonucleotide probe for 18S rRNA. The latter step was performed to correct for any small loading variability between different lanes in the same blot. The hybridised blots were exposed to photographic film at −70°C[11] and the signals obtained were measured by scanning laser densitometry (Cromoscan 3, Joyce-Loebl, Gateshead, UK).

PROBES

The probes for TNF-α and IL-1β were human cDNA and were kindly provided by Dr Alan Shaw, Glaxo Institute of Molecular Biology, Geneva, Switzerland. The TNF-α probe was 1130 hp long and was cloned in the Pst site of the pGEM-1 vector; IL-1β cDNA was 331 bp in length and was cloned in the EcoR1/HindIII site of pGEM-2.

The IL-8 probe used was a 492 base sequence from exon 4 of the IL-8 gene and comprises the last seven base pairs of the coding sequence of IL-8 and 485 base pairs of 3′ non-coding sequence[12] (Genbank accession no M28130). A non-coding region was chosen for use as a probe because coding regions may be conserved between different members of gene families, and there might be a slight risk of obtaining false signals from a coding sequence probe. The non-coding sequence selected showed no significant homology with other entries in the Genbank database. It was cloned by amplifying human genomic DNA with the primers CAAGAACCC-TACTTTCC and AGTGGAACAAGGAC-TTG, cleaving at EcoR1 and BamH1 sites present in the IL-8 sequence[12] and cloning into appropriately restricted M13. The identity of the sequence was confirmed by sequencing and the insert was excised from the phage DNA for use as a probe.[13] The 18S rRNA probe was an oligonucleotide complementary to the 3′ terminus of rRNA, terminally labelled with ^{32}p.[11]

HISTOLOGY

Antral biopsy specimens were fixed in formalin, embedded in paraffin, and 2 μm sections cut and stained with haematoxylin and eosin. Three sections per specimen were scored for the presence of gastritis, according to the system of Rauws *et al.*[14 15] This scoring method ascribes scores individually to the presence of chronic inflammatory cells, neutrophils in the lamina propria and specifically in the epithelium, and epithelial erosions. A normal mucosa scores zero and the maximum score is 10.

STATISTICAL ANALYSIS

Wilcoxon's matched pairs test was used to compare differences before and after treatment. The results are expressed as medians (ranges) and $p<0{\cdot}05$ was considered to signify statistical significance.

Results

CLINICAL

All ulcers had healed at the time of follow up, and *H pylori* was successfully eradicated in all nine patients.

CYTOKINES mRNAS

The TNF-α probe hybridised with a single size of transcript, migrating in a similar position to 18S rRNA. The band was visible after two weeks' exposure. Following the eradication of *H pylori*, the median (range) TNF-α mRNA/rRNA ratio fell from 1·29 (0·65–2·73) to 0·62 (0·4–2·68), $p=0{\cdot}02$ (Fig 1 (A) and (B)). IL-8 mRNA was identified as a single band just below 18S rRNA and was visible after two weeks' exposure. After treatment to eradicate *H pylori*, the median IL-8 mRNA/rRNA ratio fell from 21·3 (5·3–50·1) to 1·14 (0–3·0), $p=0{\cdot}004$ (Figs 1 (B) and 2 (B)).

Even with 20 μg total RNA loaded in each lane and after three weeks' exposure, the hybridisation signal with the IL1-β probe was barely visible. The band was located in a similar position to 18S rRNA but we were not

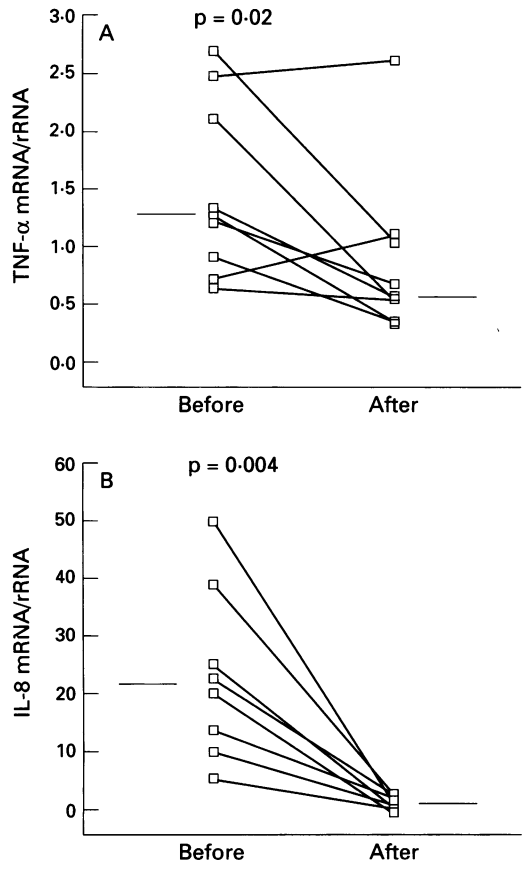

Figure 2: Changes in the expression of (A) TNF-α and (B) IL-8 mRNA before and after the eradication of Helicobacter pylori. *The results are expressed as ratios of specific mRNA/rRNA to compensate for unequal gel loading. There are only eight data points for IL-8 since the yield of RNA was insufficient for two blots in one of the patients.*

able to quantify the very weak signal by laser densitometry.

HISTOLOGY

There was a considerable improvement in the degree of gastritis after the eradication of *H pylori*. The overall gastritis score fell from a median (range) of 5·7 (3·7–7·7) to 1·0 (1·0–1·7); $p<0·01$. Neutrophil polymorphs were present in all biopsy specimens before eradication. The median neutrophil scores were 2 (1–2·7) for the lamina propria and 1·7 (0·7–3) for the epithelium. After treatment neutrophils disappeared, except in one section from one patient, which was grade 1 in the lamina propria and epithelium. The chronic infiltrate was decreased but did not disappear. The grade was consistently grade 2 before, and grade 1 after treatment. Epithelial erosions were present in one patient before, and absent in all patients after eradication of *H pylori*.

Discussion

We have shown that the antral mucosal expression of the genes encoding the cytokines TNF-α and IL-8 is decreased when *H pylori* is eradicated. Since these changes were accompanied by a reduction in the number of inflammatory cells, the findings support the role of IL-8 and TNF-α in the inflammatory processes associated with *H pylori* infection.

The reduction in IL-8 mRNA was particularly striking. The amount fell to a median of 5% of the pretreatment value after the eradication of *H pylori*. IL-8 is a peptide synthesised by the host's tissues at sites of inflammation or bacterial infection, or both.[16] The main effect of IL-8 is to attract the activate neutrophils into tissues but it is also chemotaxic for T lymphocytes.[17] Therefore much of the inflammatory infiltrate in *H pylori* infection may be due to the local production of IL-8.

Studies using human endothelial cells[18] and alveolar macrophages[19] in vitro have shown that other cytokines, including IL-1β and TNF-α, can induce expression of IL-8 mRNA. Therefore, the increased IL-8 expression in *H pylori* gastritis might result from the increased secretion of these other cytokines.

Our result show a virtual absence of IL-8 mRNA after the eradication of *H pylori*. This is consistent with the preliminary report from Atherton *et al* that tissue levels of IL-8 are virtually undetectable, unless acute inflammation is present[20]: IL-8 was not detected in chronically inflamed *H pylori* positive tissue in that study. Others, including ourselves, showed decreased but still detectable secretion of IL-8 from biopsy specimens from uninfected patients.[6–8] Many types of cells can synthesise IL-8, including endothelial cells and cells of the monocyte/macrophage lineage.[18 19] Crabtree *et al* localised IL-8 to the gastric epithelium by immunofluorescence and found that even microscopically normal mucosa expressed IL-8 peptide.[21] However, when *H pylori* was present there was also positive tissue staining in the vascular endothelium and in lymphoid aggregates.

Apparently conflicting results in this area probably reflect differences in the sensitivity of the methods used. Northern blotting allows changes in the amount of mRNA to be estimated, but is less sensitive than histological methods. The present results highlight the major fall in mucosal IL-8 mRNA which occurs on eradication of *H pylori*. Studies using in situ hybridisation would be useful to confirm the cellular origin of IL-8 in the normal and infected mucosa. *H pylori*'s cytotoxin may be involved in the stimulation of gastric mucosal IL-8 production by this bacteria. Crabtree *et al* found that cytotoxic strains stimulated a greater release of IL-8 from gastric epithelial cell lines than non-toxigenic strains of *H pylori*.[22] Presumably all our patients had toxigenic strains because all patients with ulcers are infected with this type of bacteria.[23]

After the eradication of *H pylori* we found a marked reduction in both tissue IL-8 mRNA and in the numbers of infiltrating neutrophils, consistent with the known effects of IL-8 in promoting neutrophil chemotaxis. The fall in TNF-α gene expression was less marked and was accompanied by only a partial resolution of the chronic infiltrate of cells, including those of the monocyte lineage which secrete TNF-α.

The expression of cytokine mRNAs in gastritis has not previously been investigated. Interest in cytokine expression in the gut has hitherto focussed on the inflammatory bowel diseases where changes at the mRNA level have been measured by quantitative polymerase chain reaction (PCR).[24 25] In those studies it was not possible to identify cytokine RNA transcripts in the colon by northern blotting. Indeed, TNF-α mRNA was rarely detected in the inflamed colon, even by PCR.[24] However, our experience in the stomach differs; IL-8 and TNF-α mRNAs were identified in antral biopsy specimens by northern blotting and changes in gene expression could be measured without quantitative PCR – a method we have been reluctant to employ because of problems in achieving sufficient reproducibility.

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