Mucosal tumour necrosis factor α and interleukin-6 in patients with *Helicobacter pylori* associated gastritis

J E Crabtree, T M Shallcross, R V Heatley, J I Wyatt

Abstract

The production of tumour necrosis factor α (TNF α) and interleukin-6 by human antral mucosa during short term culture in vitro has been measured by enzyme linked immunosorbsent assay. TNF α and interleukin-6 concentrations in culture supernatants were significantly greater (p<0·001) in patients infected with *Helicobacter pylori*, all of whom had chronic gastritis, than in patients who were *H pylori* negative with histologically normal gastric mucosa. Among *H pylori* colonised patients, TNF α concentrations were significantly higher in those with active gastritis and neutrophil infiltration into the epithelium than in those with inactive gastritis. In contrast, interleukin-6 concentrations were raised in both active and inactive gastritis. This study shows that *H pylori* gastritis is associated with increased gastric mucosal production of TNF α and interleukin-6 and that the nature of the mucosal cytokine response varies with the immunohistology of the disease. Inflammatory cytokines generated locally within the gastric mucosa could be relevant to the gastric physiology of *H pylori* infection.

*Helicobacter pylori* is a recently identified bacterium which colonises the human gastric epithelium. Infection is strongly associated with non-autoimmune chronic gastritis and peptic ulcer disease. While both a local and a systemic humoral response to *H pylori* is evident in infected patients, little is known about mucosal cellular immune responses to *H pylori*. Mucosal T lymphocyte density and epithelial expression of HLA-DR and secretory component are all increased in non-autoimmune gastritis. In addition, increased epithelial expression of the antibacterial components lysozyme and lactoferrin occurs.

Tumour necrosis factor α (TNF α) is a cytokine with pleiotropic functions produced mainly by activated macrophages. In addition to its oncolytic activity, TNF α has been shown to activate neutrophils, promote T and B cell proliferation, and modulate endothelial cell surface antigens. Several microbial agents induce TNF α secretion and a potent stimulator is the lipopolysaccharide of Gram negative bacteria. Recent studies have shown that lipopolysaccharide also stimulates interleukin-6 secretion, serum concentrations being raised in Gram negative septicaemias and after endotoxin or TNF α administration. Interleukin-6 was originally identified as a cytokine which induced terminal maturation of B cells, and thus was called B cell stimulatory factor. It is an important immunoregulatory molecule as well as having effects on non-immune cells. Interleukin-6 is produced by a variety of lymphoid and non-lymphoid cells including activated macrophages, fibroblasts, keratinocytes, and endothelial cells. Local increases in interleukin-6 have been associated with bacterial infections both at mucosal and non-mucosal sites.

As several microbial agents stimulate TNF α and interleukin-6, mucosal production of these cytokines may be induced by infection with *H pylori*. In this study we investigated in vitro the production of these two cytokines by normal human gastric mucosa and inflamed gastric mucosa colonised with *H pylori*. In addition, we examined the relation between mucosal secretion of TNF α and interleukin-6 and the histopathology of gastritis.

Methods

Patients

Forty three patients with dyspepsia (mean (SD) age 47·2 (15·7) years, range 20–78) were studied, none of whom was receiving non-steroidal anti-inflammatory drugs or bismuth or had had antibiotics recently. The project was approved by the Clinical Research (Ethics) Committee of the Leeds Eastern Health Authority and informed consent was obtained from all patients. Multiple biopsy specimens were obtained during upper gastrointestinal endoscopy from adjacent sites of the gastric antrum for in vitro culture and histology. The presence and severity of gastritis were assessed according to the criteria of Whitehead et al by one pathologist. Active gastritis was characterised by the presence of intraepithelial neutrophils. The gastritis was designated inactive if neutrophils were absent. Reflux gastritis was assessed according to Wyatt and Dixon. *H pylori* was identified histologically by a modified Giemsa stain. Seropositivity for *H pylori* was determined by an *H pylori* specific IgG enzyme linked immunosorbent assay (ELISA) using an ultracentrifuged sonicate antigen preparation as the antigen. Patients infected with *H pylori* were defined by histological or serological positivity or both.

In Vitro Culture

Biopsy specimens for culture were immediately placed into medium consisting of RPMI 1640 (Flow Laboratories, Rickmansworth, Herts) supplemented with 10% fetal calf serum (Sera Lab, Crawley). Initial experiments with antral biopsy specimens determined the optimal culture conditions, as well as the optimal medium, optimal concentration of antibiotic, optimal concentration of antibiotics and culture duration to permit detectable concentrations of cytokine secretion. Based on initial
results the following culture procedure was adopted. Four specimens were cultured in 1 ml of culture medium at 37°C in a 5% CO₂ humidified incubator for 24 hours. At the end of culture, supernatants were collected, centrifuged at 10 000 g, and stored at −70°C until assayed and the specimens homogenised in 2 ml 3-3 mmol/l CaCl₂. Aliquots of the homogenate were assayed for total proteins by a modified Lowry method.²¹ No differential viability of tissue from patients with or without gastritis was observed histologically. Antral biopsy specimens from eight further patients were homogenised in 1 ml 3-3 mmol/l CaCl₂ for preculture assessment of tissue TNFα in homogenate supernatants.

TNFα ELISA
Concentrations of TNFα in culture supernatants and biopsy homogenate supernatants were determined by an ELISA (T Cell Sciences, Cambridge, MA) using two murine non-competing monoclonal antibodies to human TNFα. The second layer anti-TNFα monoclonal was horseradish peroxidase conjugated and bound antibody was detected using the substrate o-phenylenediamine. Samples were assayed in duplicate and the concentration of TNFα was calculated from a standard curve of recombinant TNFα (T Cell Sciences). The ELISA sensitivity was 10 pg TNFα/ml and the assay has no cross reactivity with interleukin-1, interleukin-2, or TNFβ. Interassay variability was less than 10%.

INTERLEUKIN-6 ELISA
Interleukin-6 in culture supernatants was measured by ELISA (Research and Diagnostic Systems, Minneapolis, MN). Culture supernatants in duplicate were incubated in microtitre wells coated with a murine monoclonal antibody specific for interleukin-6. Bound interleukin-6 was detected with a peroxidase conjugated goat polyclonal antibody specific for interleukin-6. After substrate development the sample concentration of interleukin-6 was determined from a standard curve obtained by assaying serial dilutions of recombinant interleukin-6 (Research and Diagnostic Systems). The ELISA sensitivity was 25 pg/ml and the assay has no cross reactivity with interleukin-1α, interleukin-1 β, interleukin-2, or TNFα. Interassay variability was less than 10-7%.

STATISTICS
Culture supernatant TNFα and interleukin-6 concentrations were expressed as pg/mg biopsy.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Prevalence of chronic gastritis and H pylori in antral mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H pylori present</td>
</tr>
<tr>
<td>Chronic active gastritis</td>
<td>21</td>
</tr>
<tr>
<td>Inactive chronic gastritis</td>
<td>8</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Reflux gastritis</td>
<td>0</td>
</tr>
</tbody>
</table>

*Serologically positive for H pylori.

Figure 1: Concentration of TNFα in 24 hour culture supernatants of antral mucosa of patients with and without H pylori associated gastritis. Negative v positive, p<0.001.

Figure 2: Concentration of interleukin-6 (log scale) in 24 hour culture supernatants of antral mucosa of patients with and without H pylori associated gastritis. Negative v positive, p<0.001.

Results
Table I shows the prevalence of chronic gastritis and H pylori infection in the antral mucosa of the patients studied. Of the 43 patients, 29 were histologically positive for H pylori, all of whom had chronic gastritis. Two patients with inactive chronic gastritis were histologically negative for H pylori but serologically positive. Eleven of the remaining 12 histologically negative patients had normal antral mucosa and one had reflux gastritis.

TNFα production by antral mucosa was significantly greater (p<0.001) in patients with H pylori associated gastritis than in H pylori absent.
Mucosal tumour necrosis factor \(\alpha\) and interleukin-6 in patients with Helicobacter pylori associated gastritis

**TABLE 11**

<table>
<thead>
<tr>
<th></th>
<th>Active chronic gastritis</th>
<th>Inactive chronic gastritis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF (\alpha):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (pg/mg)</td>
<td>43</td>
<td>10*</td>
</tr>
<tr>
<td>Range</td>
<td>10–187</td>
<td>10–65</td>
</tr>
<tr>
<td>No</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td><strong>Interleukin-6:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (pg/mg)</td>
<td>103</td>
<td>124</td>
</tr>
<tr>
<td>Range</td>
<td>50–1520</td>
<td>25–1316</td>
</tr>
<tr>
<td>No</td>
<td>21</td>
<td>9</td>
</tr>
</tbody>
</table>

*p<0.05.

negative patients (Fig 1). The median (SEM) concentration of TNF \(\alpha\) in culture supernatants of colonised patients was 38 (7.8) pg/mg (range 10–187, n=26). TNF \(\alpha\) was detected in only one *H pylori* negative patient with histologically normal antral mucosa. Median preculture TNF \(\alpha\) concentrations in supernatants of homogenised antral biopsy specimens of *H pylori* colonised patients was 13.4 (7.3) pg/mg (range 10–46, n=5).

Interleukin-6 production by cultured antral mucosa of *H pylori* positive and negative patients is shown in Figure 2. Significantly higher (p<0.001) concentrations of interleukin-6 were present in culture supernatants of *H pylori* positive patients, median concentrations being 106 (70.4) pg/mg (range 25–1520, n=30) and 25 (11.8) pg/mg (range 25–165, n=12) respectively. The amount of interleukin-6 produced in *H pylori* positive patients was extremely variable. The highest concentrations were found in two patients with extensive intestinal metaplasia.

The relation between antral TNF \(\alpha\) and interleukin-6 production in *H pylori* positive patients with respect to the activity of the gastritis is shown in Table II. TNF \(\alpha\) concentrations were significantly greater (p<0.05) in those with a neutrophilic response (active gastritis) than those with inactive gastritis. Only in one subject with inactive gastritis were there detectable concentrations of TNF \(\alpha\). There was no significant difference in interleukin-6 produc-

![Figure 3: Relation between interleukin-6 and TNF \(\alpha\) concentrations in culture supernatants of *H pylori* positive patients with active gastritis (p<0.001, r=0.59, n=19).](image)

**Discussion**

The results show that the cytokines TNF \(\alpha\) and interleukin-6 are produced by human gastric mucosa in patients with *H pylori* associated gastritis. Overall, about 95% of patients with chronic gastritis are *H pylori* positive. Therefore in this prospective study it was not possible to include a group of patients with *H pylori* negative gastritis as disease control subjects. The pathogenesis of chronic gastritis is currently being re-evaluated after the recognition of its close association with *H pylori*. The histological changes observed are explicable as the response of the gastric mucosa to persisting *H pylori* infection and studies of local humoral immunity support this view. Our observations on mucosal TNF \(\alpha\) and interleukin-6 production in *H pylori* associated gastritis may therefore be manifestations of the presence of bacteria induced chronic inflammation.

TNF \(\alpha\) production by mononuclear cells can be stimulated by a variety of microbial agents, bacteria and their products, parasites, and pathogenic fungi. Although lipopolysaccharide is a major stimulus of TNF \(\alpha\) secretion, stimulation by non-endotoxin microbial antigens also occurs. Whether the mucosal TNF \(\alpha\) production in *H pylori* associated gastritis results solely from stimulation by *H pylori* lipopolysaccharide, other *H pylori* antigens, or is a consequence of immune activation and augmentation by interferon \(\gamma\) is currently unclear. TNF \(\alpha\) has recently been shown to stimulate lysozyme in human mononuclear cells and secretory component in intestinal epithelial cells. Local production of TNF \(\alpha\) may therefore be partly responsible for the increased lysozyme and secretory component expression observed in chronic gastritis.

Tissue concentrations of TNF \(\alpha\) in antral mucosa of *H pylori* colonised patients were lower than those of culture supernatants, suggesting that the secreted TNF \(\alpha\) was in part de novo synthesised. TNF \(\alpha\) is rarely measurable systemically in humans except in, for instance, meningococcal septicaemia. Localised increases in TNF \(\alpha\) have also been found in cerebral spinal fluid in bacterial meningitis. Mucosal production of TNF \(\alpha\) confined to the site of challenge has been shown in rats after intratracheal administration of lipopolysaccharide. Mucosal sites should therefore be considered as separate compartments from the systemic circulation with respect to cytokine production.

Few studies have examined interleukin-6 production at mucosal sites, although high concentrations have been found in cerebral spinal fluid in bacterial meningitis. In mice, urinary tract infection with *Escherichia coli* results in urinary secretion of interleukin-6 and this response is controlled by the lipopolysaccharide genotype.
In lipopolysaccharide responder mice, urinary interleukin-6 remains high while infection persists, but concentrations can be reduced by treatment with some anti-inflammatory agents. Patients receiving non-steroidal anti-inflammatory drugs were specifically excluded from the present study because of the known effects of such agents on TNF α and interleukin-6 production.

Recombinant TNF α stimulates interleukin-6 synthesis in vitro from fibroblast and endothelial cells and in vivo administration rapidly induces circulating interleukin-6 in humans. The observation, therefore, that the mucosal production of TNF α was positively correlated with interleukin-6 secretion in patients with active gastritis is not unexpected. An inhibitory action of interleukin-6 on TNF α production has been described recently, suggesting that interleukin-6 may have an anti-inflammatory effect and represent the negative arm of a regulatory circuit.

The secretion of interleukin-6, however, is unlikely to be exclusively dependent on TNF α and the relation between these cytokines is complex. In addition, interleukin-6 is produced by activated T lymphocytes. In this study there was also a clear difference in the production of both in relation to epithelial neutrophil infiltration.

Recently it was established that the functional activity of neutrophils can be modified by cytokines. While interleukin-6 will augment neutrophil oxidative burst response in vitro and increase neutrophil lysosome and lactoferrin secretion, it is not chemotactic or chemokinetic for neutrophils. This is consistent with our observation, that there was no difference between concentrations in patients with inactive and active gastritis. Similarly, murine studies have shown a dissociation between interleukin-6 secretion and mucosal polymorphonuclear cell infiltration to the site of infection.

TNF α has been shown to inhibit both human neutrophil migration and, in contrast to interleukin-6, to stimulate neutrophil chemotactic factor (interleukin-8) production by human fibroblasts and endothelial cells. Our finding that TNF α secretion was significantly lower in patients with inactive gastritis, where detectable TNF α was found in only one subject, is in accordance with this. Our observations on mucosal secretion of both interleukin-6 and TNF α can therefore be correlated with the in vitro functional properties of these two cytokines and the immunopathology of H pylori associated chronic gastritis.

Infection with H pylori is associated with an initial hypochlorhydria. Several non-gastric bacterial infections reduce acid secretion, as do certain small intestinal nematode and cestode infections. It is of interest that interleukin-1, which has many properties similar to TNF α, has recently been shown to inhibit gastric acid secretion in rats. H pylori infection is also associated with hypergastrinemia. There is some evidence that gastrin release may be stimulated by gastric immune responses. TNF α has many important metabolic effects and the inflammatory cytokines generated locally in the gastric mucosa in H pylori infections may be important in modifying gastric physiology.

This study shows that the mucosal production of both interleukin-6 and TNF α are increased in H pylori associated gastritis. Further studies will be required to determine whether H pylori bacterial products stimulate this response, the cellular origin of the cytokines, and the effects of bacterial clearance on mucosal cytokine production.

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