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

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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 immunosorbent 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.¹ Infection is strongly associated with non-autoimmune chronic gastritis and peptic ulcer disease.² While both a local^{3,4} and a systemic⁵ 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-DR⁵ and secretory component^{6,7} are all increased in non-autoimmune gastritis. In addition, increased epithelial expression of the antibacterial components lysozyme^{6,7} and lactoferrin⁷ occurs.

Tumour necrosis factor α (TNF α) is a cytokine with pleiotropic functions produced mainly by activated macrophages.⁸ 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.⁸ Several microbial agents induce TNF α secretion and a potent stimulator is the lipopolysaccharide of Gram negative bacteria.⁹ Recent studies have shown that lipopolysaccharide also stimulates interleukin-6 secretion, serum concentrations being raised in Gram negative septicaemias¹⁰ and after endotoxin¹¹ or TNF α administration.¹² 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.¹³ Interleukin-6 is produced by a variety of lymphoid and non-lymphoid cells including activated macrophages, fibroblasts, keratinocytes, and endothelial cells.¹⁴ Local increases in interleukin-6 have been associated with bacterial infections both at mucosal¹⁵ and non-mucosal sites.^{10,16}

As several microbial agents stimulate TNF α and interleukin-6,^{9,15} 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 was assessed according to the criteria of Whitehead *et al*.¹⁷ by one pathologist. Active gastritis was characterised by the presence of intra-epithelial neutrophils. The gastritis was designated inactive if neutrophils were absent. Reflux gastritis was assessed according to Wyatt and Dixon.¹⁸ *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 previously described.³ 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,^{19,20} tissue to medium ratio, and culture duration to permit detectable concentrations of cytokine secretion. Based on initial

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results the following culture procedure was adopted. Four specimens were cultured in 1 ml of culture medium²⁰ 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 TNF α in homogenate supernatants.

TNF α ELISA

Concentrations of TNF α 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 TNF α . The second layer anti-TNF α monoclonal was horseradish peroxidase conjugated and bound antibody was detected using the substrate O-phenylenediamine. Samples were assayed in duplicate and the concentration of TNF α was calculated from a standard curve of recombinant TNF α (T Cell Sciences). The ELISA sensitivity was 10 pg TNF α /ml and the assay has no cross reactivity with interleukin-1, interleukin-2, or TNF β . 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-1 α , interleukin-1 β , interleukin-2, or TNF α . Interassay variability was less than 10.7%.

STATISTICS

Culture supernatant TNF α and interleukin-6 concentrations were expressed as pg/mg biopsy

TABLE 1 Prevalence of chronic gastritis and *H pylori* in antral mucosa

	<i>H pylori</i> present	<i>H pylori</i> absent
Chronic active gastritis	21	0
Inactive chronic gastritis	8	2*
Normal	0	11
Reflux gastritis	0	1

*Serologically positive for *H pylori*.

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

protein and data are expressed as medians (SEM). Statistical analysis was performed using the Mann-Whitney U test for non-parametric data.

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.

TNF α production by antral mucosa was significantly greater ($p < 0.001$) in patients with *H pylori* associated gastritis than in *H pylori*

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, $p < 0.001$.

TABLE II *TNF α and interleukin-6 secretion in H pylori positive patients with active and inactive chronic gastritis*

	Active chronic gastritis	Inactive chronic gastritis
TNF α :		
Median (pg/mg)	43	10*
Range	10–187	10–65
No	19	7
Interleukin-6:		
Median (pg/mg)	103	124
Range	50–1520	25–1316
No	21	9

* $p < 0.05$.

negative patients (Fig 1). The median (SEM) concentration of TNF α in culture supernatants of colonised patients was 38 (7.8) pg/mg (range 10–187, $n=26$). TNF α was detected in only one *H pylori* negative patient with histologically normal antral mucosa. Median preculture TNF α 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 α and interleukin-6 production in *H pylori* positive patients with respect to the activity of the gastritis is shown in Table II. TNF α 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 α . There was no significant difference in interleukin-6 produc-

tion in patients with active or inactive gastritis. There was a significant correlation between interleukin-6 and TNF α concentrations in culture supernatants of *H pylori* positive patients with active gastritis ($p < 0.01$, $r = 0.59$, $n = 19$) (Fig 3).

Discussion

The results show that the cytokines TNF α 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.²² 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.^{3,4} Our observations on mucosal TNF α and interleukin-6 production in *H pylori* associated gastritis may therefore be manifestations of the presence of bacteria induced chronic inflammation.

TNF α production by mononuclear cells can be stimulated by a variety of microbial agents, bacteria and their products,²³ parasites,²⁴ and pathogenic fungi.²⁵ Although lipopolysaccharide is a major stimulus of TNF α secretion,⁹ stimulation by non-endotoxin microbial antigens also occurs.^{25,26} Whether the mucosal TNF α production in *H pylori* associated gastritis results solely from stimulation by *H pylori* lipopolysaccharide,²⁷ other *H pylori* antigens, or is a consequence of immune activation and augmentation by interferon γ is currently unclear. TNF α has recently been shown to stimulate lysozyme in human mononuclear cells²⁸ and secretory component in intestinal epithelial cells.²⁹ Local production of TNF α may therefore be partly responsible for the increased lysozyme and secretory component expression observed in chronic gastritis.^{6,7}

Tissue concentrations of TNF α in antral mucosa of *H pylori* colonised patients were lower than those of culture supernatants, suggesting that the secreted TNF α was in part de novo synthesised. TNF α is rarely measurable systemically in humans except in, for instance, meningococcal septicaemia.³⁰ Localised increases in TNF α have also been found in cerebral spinal fluid in bacterial meningitis.³¹ Mucosal production of TNF α confined to the site of challenge has been shown in rats after intratracheal administration of lipopolysaccharide.³² 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.¹⁰ In mice, urinary tract infection with *Escherichia coli* results in urinary secretion of interleukin-6¹⁵ and this response is controlled by the lipopolysaccharide genotype.¹⁵

Figure 3: Relation between interleukin-6 and TNF α concentrations in culture supernatants of *H pylori* positive patients with active gastritis ($p < 0.01$, $r = 0.59$, $n = 19$).

In lipopolysaccharide responder mice, urinary interleukin-6 remains high while infection persists,¹⁵ but concentrations can be reduced by treatment with some anti-inflammatory agents.³³ 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 ³⁴ and interleukin-6³³ production.

Recombinant TNF α stimulates interleukin-6 synthesis in vitro from fibroblast³⁵ and endothelial cells³⁶ and in vivo administration rapidly induces circulating interleukin-6 in humans.¹² 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,³⁷ 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.¹⁴ 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.³⁸ While interleukin-6 will augment neutrophil oxidative burst response in vitro and increase neutrophil lysozyme and lactoferrin secretion, it is not chemotactic or chemokinetic for neutrophils.^{39,40} 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.³³

TNF α has been shown to inhibit both human neutrophil migration⁴¹ and, in contrast to interleukin-6, to stimulate neutrophil chemotactic factor (interleukin-8) production by human fibroblasts⁴² and endothelial cells.⁴³ 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.⁴⁴ Several non-gastric bacterial infections reduce acid secretion, as do certain small intestinal nematode and cestode infections.⁴⁴ 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.⁴⁵ *H. pylori* infection is also associated with hypergastrinaemia.⁴⁶ There is some evidence that gastrin release may be stimulated by gastric immune responses.⁴⁷ TNF α has many important metabolic effects⁹ 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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