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# TOXINOGENICITY OF *HELICOBACTER PYLORI* IN THE PATHOGENESIS OF CHRONIC GASTRITIS AND PEPTIC ULCER AND THE NATURE OF THE HOST'S IMMUNE RESPONSES

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# PREFACE

This thesis describes several studies on *Helicobacter* pylori which sought to correlate toxinogenicity and host immune inflammatory responses.

Parts of this work have been already published. Reprints are submitted with the thesis. Some of the work has also been presented to several learned societies.

The help and contribution by colleagues and others are formally acknowledged.

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List of Abbreviations

| | |
|---|---|
| *H. pylori* | *Helicobacter pylori* |
| CL | chemiluminescence |
| PMN | polymorphonuclear neutrophil |
| VacA | vacuolating cytotoxin protein |
| vacA | vacuolating cytotoxin genes |
| CagA | cytotoxin-associated protein A |
| cagA | cytotoxin-associated gene A |
| IL-8 | interleukin 8 |
| ROM | reactive oxygen metabolites |
| IgA | immunoglobulin A |

**SUMMARY**

Despite the widespread recognition of the role of *H. pylori* infection in chronic gastritis and peptic ulceration, the mechanisms by which the bacterium induces mucosal damage and the apparent different outcome in infected patients are still not well understood. It has been suggested that the toxinogenicity of *H. pylori* and the host response may be important in these respects.

Neutrophil function is an important host defence mechanism against microbial infection. It provides a major source of reactive oxygen metabolites which are known to cause tissue damage. *H. pylori* has been shown to activate neutrophils and there are variations among *H. pylori* strains in the neutrophil activating activity. It is not known if cytotoxin-producing strains of *H. pylori* are associated with the generation of an oxidative burst in neutrophils. This has been investigated in this thesis. Cytotoxin positive strains of *H. pylori* displayed an enhanced induction of the oxidative burst in non-opsonised neutrophils compared to toxin negative strains from patients with chronic gastritis only. However, some non-cytotoxin-producing strains also induced a significant neutrophil oxidative burst, suggesting that some component(s) other than cytotoxin itself may be responsible for the induction of the neutrophil oxidative burst. Nevertheless the finding that cytotoxin-producing strains of *H. pylori* induced an enhanced neutrophil respiratory burst may suggest that the neutrophil activating property may be more strongly expressed in most toxinogenic strains of *H. pylori*. The ability of some strains of *H. pylori* to produce cytotoxin and to induce the oxidative burst in neutrophils may be important in the pathogenesis of peptic ulcer disease .

Subsequent study investigated the possible component(s) of *H. pylori* which induces the neutrophil oxidative burst. It was found that soluble products of *H. pylori* could activate neutrophils to release ROM and more than one bacterial product may be involved in the activation of neutrophils. The results also showed that the neutrophil activating activity was destroyed by proteinase K, suggesting that the active components are mainly proteins. Cytotoxin and urease containing fractions from chromatography did not induce a significant CL response, suggesting that the cytotoxin and/or urease are not the active components for the neutrophil activation.

In vitro studies have suggested that toxinogenic strains of *H. pylori*, which possess CagA(cytotoxin-associated protein) and/or VacA(vacuolating cytotoxin), may induce a more potent inflammatory response, evidenced by an enhanced induction of interleukin 8(IL-8) and neutrophil oxidative burst. It is unclear whether there is any relationship between in vivo mucosal production of IL-8 and neutrophil-derived reactive oxygen metabolites(ROM) release and whether mucosal levels of IL-8 and ROM are increased in those patients infected with toxinogenic strains. It has been shown in this thesis that there is a good correlation between IL-8 concentration and ROM release in antral gastric mucosa. This suggests that local mucosal production of IL-8 in response to *H. pylori* infection does occur in vivo and may play an important role in attracting and activating phagocytes to release ROM. In line with the in vitro finding that VacA+/cagA+ strains induced an enhanced IL-8 production in epithelial cells, patients infected with VacA+ve/cagA+ve strains had significantly higher mucosal levels of IL-8 than those infected with VacA-ve/cagA-ve strains of *H. pylori*. The former group of patients also showed a significantly higher

neutrophil infiltration score than that of the latter group. CagA+ve/VacA+ve strains may represent a subgroup of strains of *H. pylori* which stimulate a stronger cytokine release in vivo and induce more severe mucosal inflammation and tissue injury. This is supported by the finding that patients infected with VacA+ve/cagA+ve strains had a higher mucosal production of ROM compared to those infected with VacA-ve/cagA-ve strains of *H. pylori*. More toxinogenic strains were isolated from patients with peptic ulcer compared to those with non-ulcer dyspepsia (NUD). The results support the hypothesis that there may be more virulent or 'ulcerogenic' strains. Enhanced mucosal production of IL-8 and ROM could be important in VacA+ve/cagA+ve strains of *H. pylori*-induced gastroduodenal inflammation and in the development of peptic ulcer disease.

Generation of mucosal IgA is an important local immune defence mechanism. *H. pylori* induces a significant IgA response in antral mucosa in infected patients. Despite the persistent local and systemic immune response, *H. pylori* is not eliminated. However, animal studies suggest the mucosal IgA antibodies may be protective. There is little information as to the relationship if any between mucosal IgA *H. pylori* antibodies and toxinogenicity of *H. pylori* and mucosal inflammation. It has been shown in this thesis that most infected patients had raised mucosal levels of IgA antibodies. While there were large variations in the level of IgA antibodies among patients, there was no significant difference between patients infected with VacA+ve or cagA+ve strains and those infected with VacA-ve or cagA-ve strains. Lack of correlation between the mucosal antibody and the toxinogenicity of *H. pylori* may suggest that differences in individual host response may be more important. The time of infection may also be relevant. The finding that the mucosa with severe infiltration

score of neutrophils displayed lower level of IgA compared to the mucosa with mild to moderate infiltration may suggest that severe inflammation could damage the integrity of the mucosa and compromise the mucosal defence. This may render the mucosa more susceptible to injury.

There have been few studies on the effect of eradication therapy of *H. pylori* on the mucosal levels of IL-8, ROM and IgA. In this thesis, 24 patients with duodenal ulcer and infected with *H. pylori* were treated with an eradication regime consisting of lansoprazole, clarithromycin and metronidazole. In 20 patients in whom *H. pylori* were eradicated, there was a significant reduction in mucosal ROM levels. There was a less marked but significant decrease in mucosal levels of IL-8. There was also a slight reduction in IgA levels. A significant improvement was seen in histological appearance with the virtual disappearance of neutrophil infiltrate in all patients who became culture-negative for *H. pylori*, and a moderate reduction in the level of mononuclear cell infiltrate. These findings support the roles of IL-8 and ROM in *H. pylori* infection. A sustained level of mucosal specific IgA might be important in preventing reinfection by *H. pylori*.

In summary, toxinogenic strains of *H. pylori* are more likely to induce a stronger host inflammatory response by inducing an enhanced neutrophil oxidative burst and an enhanced mucosal production of IL-8 and ROM, leading to a more prominent mucosal inflammatory cell reaction. More severe mucosal inflammation may damage the mucosal IgA defence, rendering the mucosa more susceptible to infection and injury. These findings may be important in the pathogenesis of *H. pylori*-related active gastritis and peptic ulcer disease.

# CHAPTER 1 **Background**

## 1.1 Introduction

Since the finding of the *Campylobacter*-like organism, now known as *Helicobacter pylori*(*H. pylori*) in 1983 by Marshall and Warren(1,2), the understanding of the pathogenesis of chronic gastritis and peptic ulcer has been greatly enhanced by the recognition of *H. pylori* as the most important cause of chronic gastritis and an important factor in the development of peptic ulcer(3-5). Evidence has come from the following — *H. pylori* infected mucosa almost always displays histological inflammation; almost all patients with duodenal ulcer and the majority of patients with gastric ulcer are colonised with *H. pylori* (6), and eradication of the organism leads to a significant reduction in histological inflammation and in the rate of ulcer relapse(7). Studies of the epidemiology, bacteriology and immunology, pathophysiology and molecular biology have significantly increased our knowledge of *H. pylori*-related gastroduodenal disease, such as chronic gastritis and peptic ulcer, although the exact mechanisms by which *H. pylori* induces gastroduodenal mucosal pathology are still not fully understood. This chapter reviews the current understanding of the pathogenesis of *H. pylori*-related chronic gastritis and peptic ulcer.

## 1.2 Chronic gastritis

### *1.2.1 Definition*

Chronic gastritis refers to the chronic inflammatory response of the gastric mucosa to infection or injury, characterised by infiltration of lymphocytes, and

plasma cells often accompanied by polymorphonuclear cells. It is usually diagnosed histologically from gastric biopsies.

### *1.2.2 Classification and pathogenesis*

Recent advances in the understanding of the pathogenesis of gastritis and especially the role of *H. pylori* have made it possible to classify gastritis more comprehensively. Previously, it was classified as type A gastritis(i.e. autoimmune gastritis), Type B gastritis('bacterial' gastritis) and type C gastritis('chemical' or reflux gastritis) which was associated with bile reflux and possibly non-steroidal anti-inflammatory drugs(NSAID)(8). A new classification scheme — the Sydney system, which was drawn up at the 9th World Congress of Gastroenterology in Sydney in 1990, provided a more comprehensive and descriptive way to classify gastritis, which included histological and endoscopic divisions(9-10). For the histological classification, it included aetiology and topographical distribution of abnormalities and morphologies.

According to different aetiologies, it could be classified into the following categories, although there is some overlap between them.

*Helicobacter pylori-associated chronic gastritis*

It is by far the commonest type of chronic gastritis(4). It was known as chronic non-specific gastritis until 1983 when Warren and Marshall highlighted its strong association with *H. pylori*(3). The mucosa of the gastric antrum is primarily affected, although extension into the body is not uncommon in long-standing cases. Histologically, there is a chronic infiltration of lymphocytes and plasma cells, often accompanied by a superimposed patchy or diffuse acute

inflammatory cell infiltrate comprising mainly neutrophils, largely confined to the superficial part of the mucosa and the surface epithelium. Focal aggregation of lymphocytes as lymphoid follicles is also a typical finding(11). When neutrophils are present, the chronic gastritis is said to be 'active', i.e. chronic active gastritis.

In this thesis, the term of chronic gastritis refers to *H. pylori*-associated chronic gastritis, unless otherwise stated.

*Auto-immune chronic gastritis*

This type of gastritis is associated with the presence of circulating auto-antibodies, nearly always to the parietal cells(present in 80-90% cases) in the gastric glands, and often to intrinsic factor also(blocking type 70%, binding type 30%). The histological features are the loss ('atrophy') of the specialised glands in the deeper zone of the body mucosa(so-called atrophic gastritis), accompanied by a mixed chronic inflammatory cell infiltrate. It is seen classically in pernicious anaemia, where defective secretion of intrinsic factor leads to vitamin $B_{12}$ deficiency. Almost invariably there is also hypochlorhydria. Patients with advanced autoimmune gastritis or pernicious anaemia are less often colonised with *H. pylori* than is seen in the general population(12,13), It is assumed that advanced autoimmune gastritis presents an unfavourable environment to *H. pylori*.

*Reflux(chemical) chronic gastritis*

The type of chronic gastritis due to persistent reflux of duodenal contents into the stomach. It has a characteristic histological appearance of foveolar hyperplasia, oedema and vasodilation in the lamina propria(14). Inflammatory

cells are scanty, in contrast to *H. pylori*-associated gastritis. This lesion is seen classically in gastric biopsies from post-gastrectomy patients, in whom duodenal-gastric reflux is common. It is now generally accepted that this type of gastritis is caused by the damaging effects of bile acid, lysolecithin and pancreatic enzymes. Similar lesions can be seen in patients receiving long-term non-steroidal anti-inflammatory drugs, suggesting that this type of gastritis represents a reaction of the gastric mucosa to chronic chemical irritation.

*Chronic lymphocytic gastritis*

This is a newly recognised variant of chronic gastritis, characterised by marked infiltration of the surface and foveolar epithelium by mature T-lymphocytes(15-17). The aetiology is unknown, but the lymphocytic infiltrate has been linked to that in the small bowel in coeliac disease. Because of the distinctive endoscopic appearances with nodules bearing a central erosion, it has been termed chronic erosive gastritis or 'varioliform' gastritis. These varioliform lesions are usually found in the corpus alone or corpus and antrum. Similar lesions may occur in the antrum in *H. pylori* -associated chronic gastritis and reflux gastritis.

*Eosinophilic gastritis*

This is a rare condition characterised by a dense infiltration of eosinophil leukocytes into the gastric mucosa(18). Peripheral blood eosinophilia and raised serum IgE levels are often present. It is thought to be basically an allergic disorder.

*Granulomatous gastritis*

This is an uncommon type of gastritis with granulomatous inflammation in the gastric mucosa. It is recognised in sarcoidosis, tuberculosis and Crohn's disease.

### *1.2.3 Helicobacter pylori-associated chronic gastritis*

*Epidemiology*

This type of chronic gastritis is common in patients with dyspepsia referred for endoscopy(19-21). The frequency increases with age(22,23) in parallel with the age-related increase in the prevalence of *H. pylori* infection. There are geographical variations in the incidence of chronic gastritis(23-26).

*Natural history*

Siurala et al(27-29) demonstrated that chronic gastritis with a variable degree of activity is a dynamic but slowly evolving process, progressing over a period of 18 years to gastritis with mild atrophy and the latter may progress to severe atrophy after many years. There may be a small number of patients with chronic gastritis who improve over time(30), but in most patients, it is usually a progressive process, leading to a variable degree of glandular atrophy.

*Pathogenesis*

Results from pathological studies and volunteer studies confirm the association between *H. pylori* and chronic gastritis(4,31), although the exact mechanism by which *H. pylori* causes gastritis is not fully established. The bacteria do not seem to invade the gastric mucosa. On close examination of biopsy specimens from patients with *H. pylori*-related gastritis, the inflammatory events are deep in the tissue, away from the area of colonisation. One theory is that the organism sheds large amounts of soluble products which diffuse into the mucosa, against which the inflammatory and possibly a local immune response are directed(32-34). Urease has been proposed as one of these products. *H. pylori* urease has been shown to be present deep in the gastric mucosa and the

protein is known to be immunogenic(35). The presence of neutrophils in the mucosa could either be a direct consequence of some bacterial products which are chemotactic or be the result from other immune activities occurring concurrently in the mucosa, such as the production of pro-inflammatory cytokines(36-38). Accumulation of chronic inflammatory cells(i.e. lymphocytes, plasma cells and macrophages) is thought to be the result of an immune response to bacterial antigens.

## 1.3 Peptic ulcer

### *1.3.1 Aetiology*

The aetiology of peptic ulceration is still poorly understood. It has been considered to be multifactorial, involving environmental and genetic factors and has been generally accepted as a consequence of an imbalance between aggressive factors and mucosal defence mechanisms. Aggressive factors include gastric acid, pepsin, *H. pylori* infection, bile acids, NSAIDs and alcohol. Mucosal defence factors comprise mucus, bicarbonate, epithelial cells, mucosal blood flow and prostaglandins. The fact that most patients with duodenal ulcer are colonised with *H. pylori* and had higher gastric acid output suggested that these two factors are important in the aetiology of duodenal ulceration. In patients with gastric ulcers, gastric acid secretion is not increased, suggesting factors other than acid secretion, such as NSAID use, *H. pylori* and a weakened mucosal barrier are involved. The following section reviews the major factors contributing to peptic ulcer formation and their relationship to *H. pylori*.

### *1.3.2 Gastric secretion*

*Gastric acid*

Hydrochloric acid is produced by parietal cells which are mainly distributed in the body of the stomach. The numbers of parietal cells vary from person to person. The physiological functions of gastric acid include inactivation of microorganisms and activation of pepsinogen. Hydrogen($H^+$) ions are actively secreted by an $H^+/K^+$ ATPase on the canalicular membranes of the parietal cell. All stimulatory mechanisms finally activate this pump(39). Stimulators of acid secretion include histamine, gastrin and acetylcholine. Histamine stimulates acid through $H_2$ receptors on the parietal cell. Gastrin may stimulate acid secretion by releasing histamine through gastrin receptors on mast cells(40-41). Acetylcholine also stimulates secretion through cholinergic receptors both on mast cells and parietal cells. The presence of gastric acid has been considered to be essential for the development of duodenal ulcer.

*Gastrin*

Gastrin is produced by G cells in the antrum. It stimulates acid secretion and has a trophic effect on mucosal cells. A negative feedback mechanism stimulates secretion when pH rises and inhibits secretion with antral acidification. Somatostatin may be important in the control of gastrin secretion(42, 43). Protein in food and gastric distension stimulates gastrin release(44). *H. pylori* infection, both in normal healthy subjects and in patients with duodenal ulcer, is associated with hypergastrinaemia. When compared with *H. pylori*-negative healthy subjects, *H. pylori*-positive individuals have a modest elevation of serum gastrin in the fasting state and a quite substantial elevation after a meal or stimulation with gastrin releasing peptide(GRP)(45-49). Eradication *H. pylori* is followed by a complete resolution of the hypergastrinaemia(50-52).

Hypergastrinemia in Zollinger-Ellison syndrome patients results in high rate of secretion of gastric acid and pepsin, which overwhelms the mucosal protective factors, resulting in peptic ulceration.

*Somatostatin*

Somatostatin secreted from antral D cells is the main inhibitor of exocrine and endocrine secretion by the stomach. D cells in the gastric antrum are stimulated by luminal acid to release somatostatin which then inhibits adjacent G cells(42,43) and therefore inhibits acid secretion. It was found that antral somatostatin concentrations and D-cell density are significantly lower in *H. pylori*+ve patients with duodenal ulcer than in *H. pylori*-ve subjects(53-56). After eradication, antral somatostatin levels and D cell density return to the normal range, suggesting that the hypergastrinaemia found in *H. pylori*-positive patients with duodenal ulcer is due to a deficiency of antral D-cell somatostatin, which normally inhibits the synthesis and release of gastrin(57).

*Pepsinogens*

These proenzymes are produced in chief cells and mucus cells, and are converted to the active pepsin, in the presence of acid and by pepsin-induced autoactivation. There are two major groups of pepsinogens(58). Type I pepsinogens are secreted by the chief cells in the body mucosa and are encoded on chromosome 11. It can be detected in blood which correlates moderately well with acid output(59). Type II pepsinogens are also secreted from mucus glands in the antrum, duodenum and cardia in addition to the chief cells. Pepsinogen may be under the same control as gastric acid; histamine and gastrin stimulate pepsinogen secretion(60).

*Effect of H. pylori on gastric secretion*

A complicated relationship may exist between *H. pylori* and gastric acid secretion. In some patients, such as those with duodenal ulcer, *H. pylori* increases acid secretion , possibly by a decrease in the expression of gastric mucosal somatostatin and the subsequent increase in gastrin release(57). The lack of somatostatin probably explains why acid secretion is not inhibited properly during fasting and when the gastric pH is low. This may contribute to duodenal ulcer formation.

*H. pylori* has also been linked with decreased acid secretion during primary infection(61) and also in the long term by producing atrophy(62). *H. pylori*-related decreases in acid secretion in some patients have been postulated to increase the risk of gastric cancer by allowing bacteria which produce carcinogens to persist in the gastric lumen(63).

It is unclear as to why *H. pylori* increases acid secretion in some patients, while in other patients it decreases acid secretion and causes atrophy.

### *1.3.3 Mucosal defence*

*Mucus-bicarbonate barrier*

Gastric mucus consists of glycoproteins which associate by hydrogen binding to form an adherent lubricating gel over the surface epithelium(64). Mucus is secreted by surface epithelial cells, which acts as a partial barrier to diffusion of small molecules such as acid, and a more effective barrier to macromolecules and bacteria. The surface epithelial cells also secrete sodium bicarbonate, which, together with mucus can maintain a epithelial cell surface pH of 7 in the face of a luminal pH of 2(65,66). This barrier could be dissipated by certain

agents such as aspirin or bile acids. There have been limited studies in vitro suggesting that *H. pylori* can degrade mucus(67,68); it has been shown that the mucus layer in infected patients was thinner than that found in uninfected persons(69).

*Surface epithelial cell barrier*

An intact epithelial cell layer is thought to be important in mucosal defence and it has been suggested that this barrier is formed by the hydrophobic membrane of the epithelial cells, made of waxy phospholipids(70). It was shown that the hydrophobicity of isolated gastric biopsies is altered in *H. pylori* infected patients(71,72).

*Mucosal blood flow*

Blood flow is important for maintainance of mucosal pH by clearance of back-diffusing acid. Vasoconstriction predisposes the mucosa to injury induced by bile and other agents(73).

*Prostaglandins*

Inhibition of prostaglandin synthesis is considered to be the main mechanism of NSAID-related peptic ulcer. Prostaglandins protect gastroduodenal mucosa probably by maintaining mucosal blood flow(73); by increasing serosal to mucosal water flux(74), which may protect mucosa by dilution of mucosal concentration of noxious substances; and by providing direct protection of isolated gastric glands and epithelial cells against ethanol or NSAIDs(75). There is little data on the relationship between *H. pylori* infection and mucosal levels of prostaglandins.

### *1.3.4 Gastric metaplasia and duodenitis*

Gastric metaplasia is a term applied to the presence of gastric-type mucus-secreting cells in the surface epithelium of the duodenum. It is thought that the metaplastic cells originate from the Brunner's glands(76). Gastric metaplasia is distributed in a patchy manner in the duodenum, but is maximum in the first part(77,78). The incidence of gastric metaplasia increases with age(77). It is more common in men than in women, but this difference can be attributed to higher acid output in males(77). Gastric metaplasia is a non-specific response to injury and inflammation, and may be seen in Crohn's disease of the duodenum(79). The most common cause is excess acid reaching the first part of the duodenum. This has been proven in animal experiments with induced hyperchlorhydria(80-83) and by the correlation between the presence and extent of the metaplasia and maximal acid output in human subjects(84). Some studies suggest that *H. pylori* infection may also contribute to the extent of gastric metaplasia by inducing an increase in gastric acid output(85,86).

Active duodenitis is now accepted to be a precursor of duodenal ulceration(87). It has been suggested that there is an association between gastric metaplasia of the duodenum, *H. pylori* infection in the metaplastic area and the subsequent development of active chronic inflammation in the duodenum, i.e. duodenitis(88). In this hypothesis, acid-induced gastric metaplasia and *H. pylori* infection are essential prerequisites for the development for duodenitis, which may lead to duodenal ulceration.

### *1.3.4 Role of H. pylori*

Unequivocal evidence, especially that in which eradication of the bacterium leads to the cure of ulcers(89), supports the concept that *H. pylori* is an

important pathogenic factor in peptic ulceration, although the mechanisms by which *H. pylori* predisposes to ulcer formation are still not fully understood. Only a small number of infected patients develop peptic ulcer, despite a substantial number of people in the general population who are infected with the bacterium. The current understanding is that both bacterial virulence factors of *H. pylori* and host response are important in the pathogenesis of peptic ulcer.

*Virulence factors of H. pylori*

*H. pylori* cytotoxin and CagA protein(cytotoxin-associated protein) are two virulence products linked to peptic ulcer, especially duodenal ulcer. Although the *H. pylori* genome shows great diversity(90), the majority of strains can be grouped into two types. Type I strains possess cytotoxin(encoded by vac A gene) and expressing cytotoxin-associated protein(encoded by cagA gene), and are more frequently found in patients with peptic ulcer. Type II strains do not express these proteins and are less frequently found in peptic ulcer patients(91-93).

It has also been suggested that higher antral bacterial density is important in duodenal ulcer formation, although the determinants for *H. pylori* density is unclear(94).

Another hypothesis regarding the development of duodenal ulceration is that gastric metaplasia in the duodenum, in response to higher acid output, precedes *H. pylori* colonisation in the metaplastic area; duodenitis develops and eventually leads to ulcer formation(88,95).

*Host factors*

There are many more people infected with the putative ulcerogenic strains of *H. pylori* than the number of people with ulcers. Some genetic component(s) of the host's inflammatory-immune response to *H. pylori*, possibly in concert with an environmental factor(s) may also be important in the development of peptic ulcer disease. In animal studies, large variations in the severity of disease were observed after infection of different inbred strains and congenic mice with a single isolate of *H. felis*, suggesting that the difference in host response may affect the disease outcome following *Helicobacter* infection(96). However, little is known about the human genetic basis for the apparent differences in clinical outcomes.

## 1.4 Helicobacter pylori

### *1.4.1 Introduction*

*H. pylori*, once known as Campylobacter pylori, was first isolated in 1983 by Marshall and Warren(1,2). It is a Gram-negative microaerophilic spiral bacterium that resides in the stomachs of human and other primates. Recent studies have established associations between *H. pylori* infection and chronic gastritis and peptic ulcer disease(3-7). *H. pylori* infection has also been linked with an increased risk for the development of mucosa-associated lymphoid tissue(MALT) lymphoma(97-100) and possibly gastric carcinoma(101-104).

### *1.4.2 Epidemiology*

*Prevalence*

Serological studies have shown that the prevalence of *H. pylori* infection increases with age(105-110), although some data suggests that the infection is acquired primarily in childhood and the incidence is higher in younger

children(111-113). Lower socio-economic status has been found to be associated with a higher prevalence of *H. pylori* infection(110,114-118). *H. pylori* infection is more common in dyspeptic patients. The prevalences have been reported as 45-87% in non-ulcer dyspepsia patients(119-123), 90-100% in duodenal ulcer patients (124,125) and 60-100% in those with gastric ulcers(126-128).

*Source and mode of transmission*

Both the source of *H. pylori* infection and the mode of transmission are largely unknown. *H. pylori* is generally considered to be a human pathogen, but the isolation of *H. pylori* from macaques suggest that non-human reservoirs may exist, although it is not known if this species provides an important reservoir for human infection(129-130). The isolation of viable *H. pylori* in saliva and gastric juice of domestic cats implies that transmission from cats to humans is possible(131). The detection of *H. pylori* in dental plaque(132), saliva(133), bile samples(134) and faeces(135-136) by PCR or/and culture would support an oral-oral or faecal-oral mode of transmission. Although the exact mode of person-to-person transmission is unknown, some studies have suggested that some occupational groups may be at greater risk of acquiring *H. pylori* infection e.g endoscopists(137); adequate disinfection and cleaning of endoscopes appears to eliminate the risk of transmission(138).

### *1.4.3 Diagnosis of infection*

Since the isolation of *H. pylori* in 1983, a wide range of techniques has been established for the diagnosis of *H. pylori* infection. Although isolation and identification of the organism in endoscopic biopsies remains the 'gold

standard', some indirect methods, such as the urea breath test, allow the detection of *H. pylori* to be made without subjecting patients to endoscopy. Tests based on antibody detection in serum is also a non-invasive technique; these have played an important role in epidemiological studies.

Methods of detection of *H. pylori* can be categorised as endoscopy based tests(or invasive tests) and non-invasive techniques.

Invasive tests include histology, bacterial culture, biopsy urease testing, and some molecular techniques such PCR and DNA hybridisation. Non-invasive methods include urea breath tests and serological tests.

*Invasive methods*

*Histology*

Direct visualisation under the microscope of *H. pylori* in endoscopic biopsies by various staining methods provides reliable and sensitive detection of the organism. The Warthin-Starry silver stain was recommended initially, because it is sensitive and very good for visualisation of the bacterium(139). However, the method is relatively complicated and time-consuming. The conventional haematoxylin and eosin stain is very well suited for assessing and grading inflammation, but less reliable for detecting *H. pylori*, because the organism is less easily distinguishable with this stain. Giemsa stain is considered to be a good choice, because of its simplicity, speed and low cost(139). Cresyl fast violet staining has also been used and proven to be convenient and reliable(140). Immunohistochemical(141-142) and immunofluoresent methods(143) have also been regarded as reliable for detection of *H. pylori* in

fresh biopsies, frozen sections or in formalin-fixed tissue. However, these techniques are relatively more complicated and more expensive.

*Culture of H. pylori*

Isolation and characterisation of the organism is the "gold standard" for identification of *H. pylori*, although it needs some special equipment and is relatively time-consuming. Isolation of *H. pylori* by culture is particularly valuable for typing the organism and for determining its sensitivity to antibacterial agents. It is also important for investigating the virulence factors of *H. pylori* including several antigens used for the production of serological assays and possible vaccines.

*Biopsy urease testing*

A very simple way of detecting *H. pylori* is to place the endoscopic biopsy specimen into a urea-containing medium, and based on the organism's ability to produce large amounts of urease, a distinctive colour change usually can be seen within hours. It is both sensitive and specific(144-146).

*Molecular techniques*

These methods include PCR tests and DNA hybridisation, using *H. pylori* specific primers and DNA probes. They are very sensitive and fairly specific(147,148). PCR allows the *in vitro* production of large amounts of specific DNA. Application of PCR technique to *H. pylori* research has not only provided a new approach to diagnosis of *H. pylori* infection, but also allowed the detection of specific genes such as cagA, fingerprinting of individual strains, cloning and sequencing of important genes(149).

*Non-invasive methods*

*Urea breath tests*

In an infected patient, *H. pylori* urease hydrolyses urea, labelled with $^{13}C$ or $^{14}C$, to release labelled $CO_2$, which is exhaled and can be detected. The urea breath testing methods are both sensitive and specific for active *H. pylori* infection (150), which is especially useful in assessing the efficacy of eradication therapy(151).

*Serological tests*

Based on the systemic immune response elicited by *H. pylori* infection, various serological tests have been developed to detect specific antibodies to the bacterium. The IgG isotype has been used more widely and more reliable than IgA(152). In general, enzyme-linked immunosorbent assay(ELISA) is considered to be more sensitive than other techniques such as agglutination and complement fixation(153). Most of the current commercial ELISA kits using partially purified antigens containing urease, give a fairly high sensitivity and specificity(154,155). However, comparisons are sometimes difficult between different studies and the cut-off values are difficult to standardise for different populations(156,157). It is less reliable in diagnosing active infection, because the presence of antibody may indicate a past exposure to the organism. It is more useful in epidemiological studies and may be useful in monitoring the long-term effect of treatment. Several studies have shown that after eradication, serum IgG and IgA antibodies gradually fall within six months(158).

### ***1.4.4 Treatment of H. pylori infection***

It is accepted that patients with duodenal ulcer and gastric ulcer with *H. pylori* infection should be treated with eradication therapy(159,160). The combination

of an antisecretory drug, usually a proton pump inhibitor, with two antibiotics has been accepted as an highly effective regimen for *H. pylori* eradication. The most commonly used regimens include one proton pump inhibitor(either omeprazole or lansoprazole), and two antibiotics from among clarithromycin, a nitroimidazole(metronidazole or tinidazole) and amoxycillin. The new short-term and low dose triple therapy regimen has been proven to be as effective as a longer one, with better compliance, fewer side effects and lower cost(161,162).

### *1.4.5 Virulence factors of H. pylori*

*H. pylori* produces several enzymes and exotoxins, which have been suggested as being important in inducing gastroduodenal mucosal damage. Colonisation of *H. pylori* in the gastric mucosa is accepted as a prime determinant of pathogenicity, considering that the acidic gastric environment is hostile to most microbial species. Several factors have been suggested to be important in helping colonisation of *H. pylori* including the urease and various adhesins.

*Urease*

Urease is an highly active enzyme with a molecular mass of 600Ka and is composed of two subunits, UreA(26.5KD) and UreB(60.3KD)(163,164). The native protein enzyme consists of six copies each of UreA and UreB.

Studies have also established the important role of urease in the pathogenesis of *H. pylori*. Production of the enzyme is essential for successful colonisation of the gastric mucosa(165) and triggers a strong serum immune response(166,167). All the gastric *Helicobacter* species isolated so far have a similar urease enzyme, suggesting that it is important in colonisation(168-170). Direct evidence has been provided by an animal study in which a urease-negative

variant of *H. pylori* failed to colonise gastric mucosa in gnotobiotic pigs(165). The urease helps *H. pylori* to survive in gastric acid by production of ammonia leading to its penetration of the mucus surface and colonisation within an alkaline environment(171).

In addition to its beneficial effect on survival, urease itself could cause direct tissue damage. Some studies have suggested that ammonium hydroxide, generated by urea hydrolysis, contributes significantly to histological damage(172-174). Urease activity may also cause tissue damage through interaction with the immune system. Studies have suggested that urease can act as chemotactic factor and activator for monocytes and neutrophils, resulting in the release of inflammatory cytokines and reactive oxygen metabolites to cause mucosal injury(175,176).

*Adhesins and/or receptors.*

The findings that *H. pylori* has a very specific association with gastric-type epithelium suggests the presence of adhesin molecule(s) and/or specific receptor(s) on gastric mucosal cells. Several potential adhesins and receptors have been identified, including haemagglutinins, urease and flagellin on the bacterium and laminin on the gastric cell(177). Studies have also suggested that the Lewis b blood group antigen is a *H. pylori* receptor on the gastric surface(178-180).

*Phospholipases*

Phospholipase A2 and C are invariably detected in strains of *H. pylori* (181,182). Because the phospholipids are important components in the gastric mucosal defence, the degradation of these components by phospholipases could

damage mucosal integrity(183). It has also been suggested that *H. pylori* phospholipases can disrupt the normal phospholipid bilayer of the epithelial cell membrane and liberate arachidonic acid, which is then converted to leukotrienes and other eicosanoids(184). These compounds may change membrane permeability and mucus discharge.

***Catalase***

Catalase acts on hydrogen peroxide to form water and oxygen.

$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2^-$$

*H. pylori* has very high catalase activity, 3-10-fold higher than that of most other bacteria. This could be related to adaptation of the enzyme to an environment rich in toxic oxygen metabolites(185). So it could be considered to be a virulence factor which may protect the bacterium from the damaging effects of reactive oxygen metabolites produced by phagocytes(186-188).

***Superoxide dismutase***(SOD)

Superoxide($O_2^-$) is destroyed by SOD as shown in the following reaction:

$$2\,O_2^- + 2H \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Like catalase, SOD is also involved in catalyzing the breakdown of superoxide radicals. So it may also protect the bacterium against killing by neutrophils in vivo.

*Cytotoxin and CagA protein*

The vacuolating cytotoxin is one of the first toxic products identified from *H. pylori*. The toxin is excreted into the culture medium and has been shown to

cause vacuolation in several cultured cell lines. It was shown that cytotoxic strains were more frequently found in patients with duodenal ulcer than in patients with chronic gastritis alone(189).

CagA is another highly immunogenic product of *H. pylori*, which was shown to be linked with peptic ulceration and its expression is closely associated with cytotoxin production(91-93).

***1.4.6 H. pylori cytotoxin and CagA protein***

Leunk et al first described the cytotoxic effect of *H. pylori* and found activity in about 50% of the strains from different parts of the world(190). Broth culture supernatants of the bacterium induced a non-lethal vacuolating effect on tissue culture cell lines including Vero cell and HeLa cells. Figura et al(189) found that cytotxic strains were more frequently found in patients with peptic ulceration than those with chronic gastritis alone and suggested that cytotoxinogenic strains were associated with serious gastroduodenal disease more often than non-toxinogenic strains. This finding was later supported by others(191,192). In addition, toxinogenic strains were more frequently found in areas where there was a high prevalence of gastric atrophy and gastric cancer(193-195). Therefore testing strains of *H. pylori* for toxinogenicity may be helpful in determining the risk of developing peptic ulcer and gastric malignancy in different populations and in determining which group of infected patients should be treated.

*Nature of toxin*

Cover and Blaser(196) have shown that the native cytotoxin of *H. pylori* is a protein aggregate with a molecular weight ≥970kDa. Sodium dodecyl sulfate-

polyacrylamide gel electrophoresis(SDS-PAGE) analysis showed that the protein is composed of subunits of 87kDa. The gene expressing the toxin was designated vacA. Studies have shown that the vacA gene was present in all strains of *H. pylori*(197-200) including cytotoxin-negative strains; however, the gene does not express cytotoxin in these strains. It was shown that in certain regions of the gene, there is substantial diversity(201,202). In the highly diverse middle region, sequences from cytotoxic strains form a family in which the sequences are highly related(90%) to one another. The non-cytotoxic strains form another highly related family(>90% identity in the middle region)(197,201). It was shown that the vacA genes are a mosaic, consisting of any one of three signal sequences(s1a, s1b, s2) and one of two middle-region alleles(m1, m2). In total, any *H. pylori* strain could have one of six possible genotypes; vacA genotype correlated well with cytotoxin phenotype. It was shown that s1/m1 genotypes were high cytotoxin-producers; s2m2 type strains were non-toxin producers(201).

*Activity of toxin*

Studies have shown that the toxin induces intracytoplasmic vacuole formation in cultured cell lines(190). Vacuoles are first visible around nuclei and become larger and confluent, and finally break up resulting in cell death. Ammonia and nicotine, which can cause cell vacuolation on their own, could potentiate the vacuolating activity of the toxin in vitro(203,204). This could possibly account for a higher prevalence of duodenal ulcer in patients who smoke.

The mechanism of vacuolation has been recently clarified by Papini et al(205), who demonstrated that the toxin stimulates a cellular proton pump. There are

many different ATPases at the level of the membrane and intracellular compartments. The target of the toxin is a vacuolar-type adenosine triphosphatase(ATPase) of the endocytic compartment. As a result of this stimulation, a transmembrane pH gradient is formed. Basic substances, always present in the environment, cross endocytic membranes and are protonated as they encounter an acidic environment; once protonated they remain inside since the membranes are not permeable to protonated substances, and attract water osmotically which causes swelling.

It has been shown that the vacA genotype is associated with the likelihood of having peptic ulceration; s1m1 type strains were more frequently found in patients with peptic ulceration or a past ulcer history, whereas s2/m2 genotype were much less often associated with peptic ulceration(206).

Extracts of VacA+ cytotoxic strains were shown to inhibit gastric cell proliferation in vitro by preventing epidermal growth factor from binding to its receptor on cultured cells(207). It was suggested that cytotoxic strains may interfere with the healing process of erosive and ulcerative lesions in vivo(208).

*CagA protein*

Further evidence in support of cytotoxic strains being more pathogenic comes from studies of the immune response to a 120-140kDa protein which is closely associated with the expression of cytotoxin and has been termed the cytotoxin-associated protein(CagA). The CagA protein is highly immunogenic; infection with CagA positive strains induces a high level of anti-CagA antibodies, both locally and systemically. Cover et al(209) first reported that all duodenal ulcer patients studied had serum IgG antibodies to a 128KD protein and only about

60% of patients with gastritis alone had antibodies to this protein. Crabtree et al(210) showed that gastric mucosal IgA responses to a 120KD protein was associated with peptic ulceration. It was found that about 40-60% of patients with gastritis alone were infected with CagA+ve strains compared to 80-100% of those with duodenal ulcer(209-211). In patients with gastric ulcer, the results were intermediate(209,211). It was also shown that infection with CagA+ve strains of *H. pylori* was associated with a increased risk of development of atrophic gastritis and gastric adenocarcinoma (212,213).

*The cagA gene*

The gene encoding the CagA protein was cloned and designated cagA in 1993(91,92). Unlike the vacA gene, the cagA gene is not present in all strains of *H. pylori*. The cagA gene is only present in strains expressing the CagA protein. One feature of the gene is the presence of repeated patterns. The difference in number of repeats could explain the size range of the CagA protein from 120-140KD(91).

It was shown that cagA+ve strains were associated with more severe gastric inflammation and epithelial injury compared to cagA-ve strains(210). Studies in vitro also showed that cagA+ve strains induced higher levels of interleukin-8(IL-8), a potent proinflammatory cytokine, than cagA-ve strains(214).

*Relationship between VacA and CagA*

It was shown that in about 70% strains of *H. pylori*, the expression of cytotoxin(VacA) are correlated with the possession of cagA gene(215). In a genetic study, cagA status was found to be concordant with the vacA genotype

in most strains studied. All s2(vacA genotype) strains were cagA-ve and all cagA+strains were s1(vacA genotype)(201).

### 1.5 Host responses to *H. pylori*

*H. pylori* infection induces significant local and systemic immune responses, as shown by infiltration of neutrophils, specific T-and B-cell responses, local production of proinflammatory and immunoregulatory cytokines, and both local and systemic antibodies to *H. pylori*. Recent studies have suggested that there are strain to strain variations in the induction of mucosal inflammatory responses(216).

#### *1.5.1 Neutrophils*

*Morphological and structural characteristics*

Neutrophils contain two major granule populations, primary or azurophil granules and specific or secondary granules(217). Primary granules contain acid hydrolyses, neutral proteases, myeloperoxidase, cationic proteins and lysozyme. The main function of primary granules is microbial killing and digestion. Specific granules contain lactoferrin, lysozyme, vitamin B12 binding protein and cytochrome b, and their membranes serve as a source of receptors.

As part of the host's system of defence against infection by pathogenic microorganisms, the roles of neutrophils are to seek out, ingest and kill the invading organisms. These processes involve chemotaxis, phagocytosis, degranulation, the neutrophil oxidative burst, and bacterial killing.

*Chemotaxis*

Chemotaxis is a process in which neutrophils receive signals from invading organisms or injured or dead tissue and move towards the area from which the

signal emanates. The process involves many protein molecules and inflammatory mediators including intercellular adhesion molecules(ICAM), bacterial chemoattractants and cytokines(218-220).

***Phagocytosis***

Phagocytosis is a process by which bacteria are ingested and killed by white blood cells such as neutrophils and macrophages. This is one of the major defence mechanisms by which the immune system protects man against bacterial infection.

It is a two step process involving attachment and engulfment of the phagocytic particle. Some microorganisms and inert particles may be ingested by neutrophils in the absence of serum factors but most bacteria must be coated with opsonins for attachment to neutrophils to occur. Specific IgG and complement are the major opsonic factors promoting recognition and ingestion of microorganisms by neutrophils.

Once the phagocytes have arrived at the focus of bacterial infection and come into contact with the bacterium through specific receptors, engulfment of the bacterium takes place as a result of fusion of pseudopodia which project from the plasma membrane of the phagocyte. The pseudopodia engulf the bacterium to form a vesicle called phagosome, which then buds off from the plasma membrane and lies within the cytoplasm of the neutrophils or macrophages. Once the phagosome has formed, lysosomes moves towards to it and fuse to form the phagolysosome. The lysosomal granules disappear as 'degranulation' occurs.

***Degranulation***

Degranulation is initiated before completion of ingestion. Ninety percent of the contents released from specific granules during phagocytosis can be found outside the cell. These granules appear to function principally as secretory granules. More than 50% of the contents released from primary granules during phagocytosis can be recovered from the phagosome(221). These enzymes function best at the low pH(6.0-6.5) found in phagosomes. Consequently, the contents of the primary granule appear to function principally in microbial killing and digestion.

*Oxidative burst*

The process of phagocytosis is associated with a burst of metabolic activity — the respiratory burst, which results essentially in the stepwise reduction of molecular oxygen to hydrogen peroxide. This respiratory burst is associated with a 2- to 20-fold increase in oxygen consumption compared to a resting cell and a considerable increase in glucose metabolism via the hexose monophosphate shunt.

The basic series of reactions of the respiratory burst results in (1) oxygen consumption, (2) superoxide production, (3) hydrogen peroxide production, and (4) stimulation of the hexose monophosphate shunt. The oxidative burst process is illustrated in Fig 1.

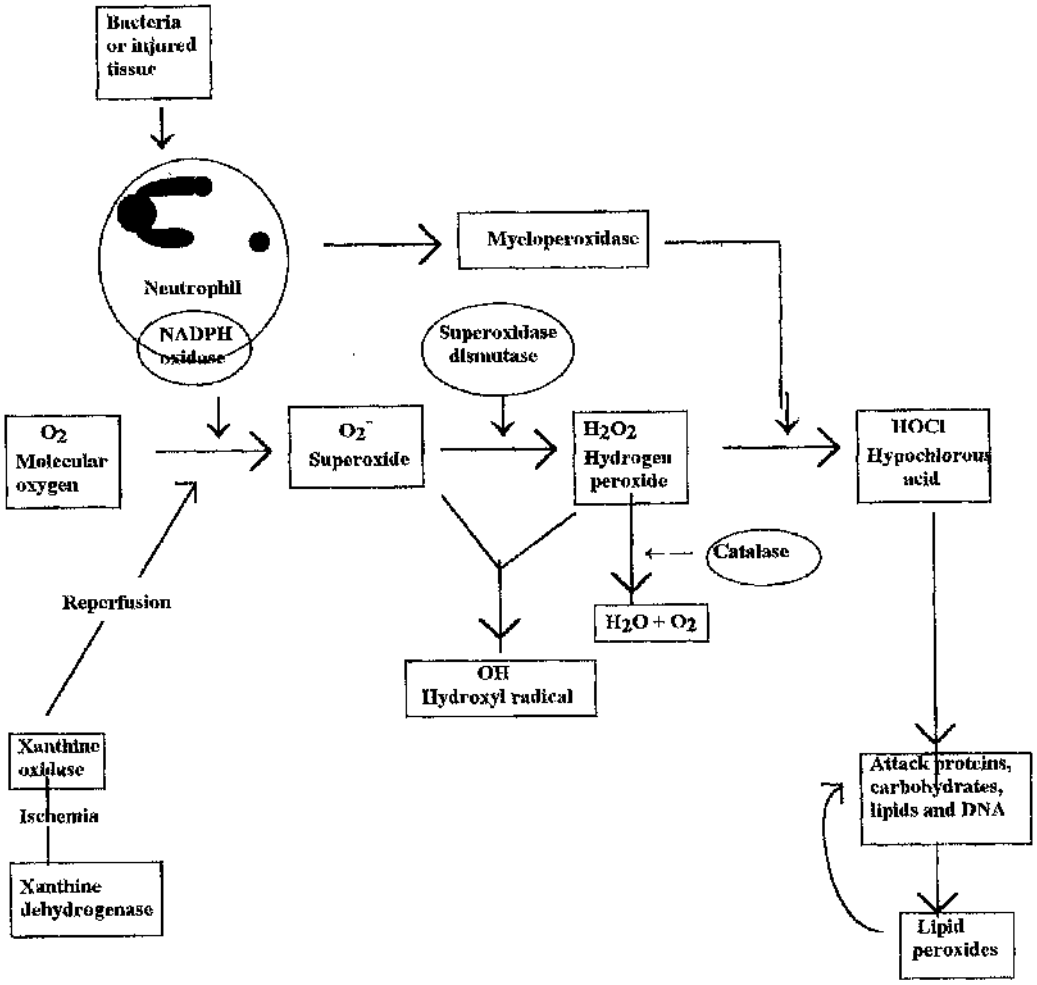


**Fig 1 Source of reactive oxygen metabolites and their metabolism**

*Microbicidal mechanisms*

The above postphagocytic events are designed to deliver the products of degranulation and the respiratory burst to the phagocytic vacuole. The phagosome plays an important role in this process because it provides a closed space in which an ingested microbe is exposed to high concentrations of toxic substances and the exposure of the phagocyte and other cells to these metabolites is minimised(222).

Oxygen dependent bactericidal mechanisms can be divided into myeloperoxidase-dependent and -independent reactions(223). The essential requirements for the myeloperoxidase-dependent mechanisms include myeloperoxidase, released from the primary granule; hydrogen peroxide, from the oxidative burst. Hydrogen peroxide has a bactericidal effect itself, but in the presence of myeloperoxidase, the potency is enhanced by 50-fold. Other oxygen metabolites including superoxide, singlet oxygen, hydroxyl radical and hypochlorous acid are also considered to have microbicidal properties(223-225). The oxygen-independent microbicidal mechanisms include lactoferrin, lysozyme, and cationic proteins. The enzymes released from the granules are important in the killing and digestion of ingested microorganisms(225,226).

*Tissue injury associated with neutrophil function*

Ordinarily, degranulation and the oxidative burst are restricted to the points of contact between the organism and the phagolysosome(227,228). There are receptor-mediated mechanisms to downregulate the oxidative process during continuous exposure to homologous stimuli(229) and to products of the oxidative burst(230). Oxidative inactivation of inflammatory

mediators(231,232), and the oxidase itself would further limit neutrophil activation and confine the toxic effects to the vicinity of the organism. However, the toxic substance of these microbicidal systems can be released and cause damage to host tissues in certain diseases associated with autoantibody formation, immune complex deposition, excessive release of inflammatory mediators, and chronic low-grade inflammation(233,234).

### *1.5.2 H. pylori and neutrophil function*

*H. pylori* infection is associated with active chronic gastritis, which is characterised by a prominent infiltration of neutrophils and mononuclear cells. Studies have shown that *H. pylori* secretes chemotactic factors, which might account for the considerable inflammatory response in gastric mucosa. Craig et al(235) reported that *H. pylori* supernatant showed chemotactic activity for neutrophils, attracting about 50% of the cell activity seen with formyl-methionyl-leucyl-phenylalanine(FMLP); monocytes were attracted with about 90% of the activity observed with FMLP. The chemotaxin was considered to be a protein(s) because it could be destroyed by proteinase(236,237). Interleukin 8 released from gastric mucosa in response to *H. pylori* infection may also contribute to the accumulation of inflammatory cells(216).

There is also evidence that *H. pylori* can activate neutrophils to produce a respiratory burst. Nogaard et al(236) showed that *H. pylori* could cause neutrophil degranulation releasing myeloperoxidase, which is a crucial component in oxygen dependent microbial killing. Several studies using chemiluminescence have also demonstrated that H. pylori could activate neutrophils to produce reactive oxygen metabolites(237). The neutrophil

activating property has been suggested to be a protein(s)(236). Mai et al(175) showed that soluble surface proteins from *H. pylori* activate monocytes/macrophages through a lipopolysaccharide(LPS)-independent as well as a LPS-dependent mechanism. It was shown that soluble surface proteins, which are rich in urease and do not contain LPS, stimulate monocytes to express HLA-DR and IL-2 receptor and to produce IL-1 and TNF. It was also shown both of these preparations induced monocyte production of superoxide($O_2^-$).

### *1.5.3 Reactive oxygen metabolites(ROM)*

*Source of ROM*

There are two possible sources of ROM production in amounts of pathological importance(238)(Fig 1): activated phagocytes (particularly neutrophils) and xanthine oxidase, formed from xanthine dehydrogenase during tissue ischemia and catalysing the formation of superoxide during reperfusion. The neutrophil membrane contains an NADPH-dependent oxidase system that is a highly efficient source of superoxide radical generation. The enzyme is normally dormant, but after phagocytosis occurs, the oxidase becomes active, resulting in the production of superoxide. The superoxide is the precursor of hydrogen peroxide, which gives rise to hypochlorous acid in the presence of chloride ions and myeloperoxidase.

*Mechanisms of damage due to oxidative stress*

ROM production during the inflammatory response is an important host defence mechanism against certain micro-organisms, but it is not always confined within the phagocytic cells(239). When produced in excess, ROM could be released to

the outside of the cell, from whence they can attack extracellular targets, causing cell/tissue damage(239,240). The mechanisms by which ROM induce cell injury include protein degradation(241), lipid peroxidation(242) and DNA damage(243).

*H. pylori and ROM production*

Studies in vitro have shown that *H. pylori* and its soluble products could activate phagocytes to produce ROM through the oxidative burst(237). Davies et al(244)also showed that *H. pylori* infection is associated with an excessive amount of ROM within infected gastric mucosa. They have also shown significantly greater free radical production in duodenal mucosal biopsies from patients with duodenal ulcer and severe duodenitis(245). ROM production correlated well with macroscopic and microscopic mucosal damage, suggesting some pathological importance of ROM. The mucosal production of ROM was associated with neutrophil infiltration, suggesting that neutrophils are the predominant source of ROM.

### *1.5.4 Cytokine response to H. pylori*

#### *Introduction*

Cytokines are proteins or glycoproteins that generally have a molecular weight less than 30kda. They generally function as intercellular messenger molecules that evoke particular biological activities after binding to a receptor on a responsive target cell. Although a variety of cells can secrete cytokines, T helper cells and macrophages are two principal producers.

Studies have shown that *H. pylori* stimulates mucosal production of proinflammatory cytokines, including interleukin 8(IL-8), IL-1, IL-6, tumour

necrosis factor(TNFα) and interferon γ(IFN-γ). Among these, IL-8 has been shown to be the most important in *H. pylori*-related inflammation. Other cytokines including IL-1 and TNFα may be important in regulating the production of IL-8(216).

***Interleukin 8***

***Source***

IL-8 originally purified from conditioned medium of endotoxin-stimulated human monocytes, is produced by a variety of cells including monocytes/macrophages, fibroblasts, endothelial cells, epithelial cells and neutrophils(246-248). Gastric epithelial cells, in common with epithelia from other mucosal sites(249,250) are a major source of IL-8(37,251). It has been shown that epithelial cells secrete IL-8 in response to bacterial entry, and suggested that epithelial cells serve as an early signalling system to host immune and inflammatory cells in the underlying mucosa following bacterial entry(249).

***Biological activity***

IL-8 belongs to the group of the α-chemokines and acts predominantly on neutrophil granulocytes(36). It is the most potent neutrophil chemotactic cytokine known so far(252). It induces degranulation, promotes the attachment and transendothelial migration of neutrophils(252,253). IL-8 is also chemotactic for T-lymphocytes(254,255). Thus IL-8 is an important determinant regulating the trafficking of leukocytes and the development of an inflammatory response(256). In contrast to the classic neutrophil chemoattractants(i.e.,

formyl-methionyl-leucyl-phenylalanine, platelet activating factor, leukotriene B4, and the fifth component of complement, C5a), IL-8 is extremely long-lived and is resistant to both high temperature and enzymatic degradation(252,257). In addition, a single stimulus will serve to induce continuing production of IL-8 protein and persisting levels of IL-8 mRNA in vitro(258). The persistence of IL-8 mRNA and protein when compared with other inflammatory cytokines reflects the potency of IL-8 as a leukocyte chemokine.

***Regulation***

Cytokines rarely act alone in vivo. Multiple cytokines are usually present at any site of inflammation, in which they may interact with each other. It has been shown that IL-1 and TNFα and LPS can upregulate IL-8 production(252,259,260). IL-4 and IL-10 inhibit the production of endothelial IL-8(256).

***H. pylori infection and Il-8***

IL-8 mRNA expression in the gastric epithelium and mucosal secretion of IL-8 from antral biopsies are increased in *H. pylori* infected patients(251,261,262). Studies have shown that expression of IL-8 in gastric epithelial cells may be upregulated by the inflammatory cytokines TNFα and γ-IFN, as well as by direct stimulation by *H. pylori* products(263). Gastric TNFα(264,265) and IL-1(266,267) message expression and protein concentration are increased in *H. pylori*-related chronic gastritis. It was shown that both *H. pylori* LPS or LPS-free surface components induced cytokine secretion from peripheral monocytes(175). It was also suggested that neutrophils may secrete

inflammatory cytokines including IL-1, TNFα and IL-8, which could further amplify the cellular response to infection(268).

In vitro studies have shown that there are strain to strain variations in their ability to induce epithelial IL-8 production. CagA/cytotoxic strains stimulated enhanced production of IL-8(269), and it was later shown that CagA positivity, rather than VacA(cytotoxin) expression is linked with IL-8 production(270). Studies using both strains naturally expressing cytotoxin and/or CagA and their isogenic mutants have shown that although CagA expression is closely associated with the induction of IL-8, neither VacA nor CagA is the direct inducer of IL-8(271). However, more recent studies suggest that the cagA gene is a marker for the presence of a group of genes(the *cag* pahtogenicity island) in type I strains, which may directly regulate the cytokine production(272).

### ***1.5.5 Humoral immune response***

#### ***Mucosal antibodies to H. pylori***

*H. pylori* infection is associated with the development of gastric lymphoid follicles which are absent in histologically normal mucosa(11). These follicles may be the source of the increased numbers of plasma cells, which produce *H. pylori* specific antibodies. Using immunohistochemical methods, *H. pylori* coated with specific IgA, IgG and IgM antibodies have been identified in gastric biopsies(273). *H. pylori* specific IgA was found by ELISA in gastric biopsy homogenates and gastric juice in infected patients(273-275). IgG plasma cells are also increased in chronic gastritis, particularly those of the IgG1

subclass(276). Lower levels of IgG, compared with IgA, were detected in gastric biopsies(277).

The role of the mucosal *H. pylori* specific antibodies in the immunopathology of *H. pylori*-related gastroduodenal disease is unclear. The mucosal IgA is likely to be important in inhibiting antigen uptake, blocking bacterial adherence and motility of the organism and also in toxin neutralisation(278). It was shown that the IgA antibodies inhibit the cell vacuolation induced by *H. pylori* cytotoxin in vitro(279). However, current information on the association between mucosal antibodies and gastroduodenal disease is limited. CagA, a highly immunogenic protein, induces a strong immuno-reactive specific IgA response and has been linked with more active gastric mucosal inflammation and peptic ulceration(210).

***Systemic antibodies to H. pylori***

Serum specific IgG, IgA and IgM can be detected in *H. pylori* infected patients(152,153). Detection of specific IgG and IgA, especially IgG, has been used in the serodiagnosis of *H. pylori* infection, although it cannot differentiate current infection or a recent past exposure to the bacterium. It is mostly used in epidemiological studies.

One study has shown an association between serum anti-*H. pylori* antibody titres and disease severity. IgG titres were shown to be correlated with the severity of inflammation both in the antrum and in the body of the stomach(280). Serum IgG responses to the 54KD heat shock protein and to the vacuolating cytotoxin were correlated with acute mucosal inflammation(281). IgA responses to whole-cell sonicate and to the cytotoxin were inversely related

to chronic inflammatory scores(281). Serum IgG antibodies to CagA has been shown to be associated with gastric atrophy, as well as peptic ulcer disease, and gastric cancer(213,282).

## 1.6 Aims of Study

Despite recent advances in the understanding of the association between *H. pylori* infection and chronic gastritis and peptic ulcer, the mechanisms by which *H. pylori* induces gastric inflammation and predisposes to ulcer formation are not fully understood. Both bacterial factors and host immuno-inflammatory responses are considered to be important in the pathology, although current information about the relationship between the two and its importance in chronic gastritis and peptic ulceration is still limited.

The studies were designed to investigate the association between the toxinogenicity of *H. pylori* and host immuno-inflammatory responses in patients with chronic gastritis and peptic ulcer. The study will seek to show —

(1) an association between the toxinogenicity of *H. pylori* and activation of neutrophils isolated from human peripheral blood.

(2) which component(s) of *H. pylori* is involved in activating neutrophils.

(3) an association between IL-8 levels and mucosal production of reactive oxygen metabolites in patients with chronic gastritis and peptic ulcer disease and the nature of their relationship to toxinogenicity of *H. pylori*.

(4) a relationship between mucosal IgA *H. pylori* antibodies and toxinogenicity of *H. pylori* and mucosal inflammation.

(5) an effect of eradication therapy of *H. pylori* on mucosal immuno-inflammatory response.

# CHAPTER 2 PATIENTS AND METHODS

## *2.1* Patients

Patients with dyspeptic symptoms and referred to the Department of Gastroenterology at Glasgow Royal Infirmary University NHS Trust for endoscopy were recruited consecutively. During the period between 1993-1994, 76 patients from whom 76 strains of *H. pylori* were isolated were entered into the study of "association of cytotoxin and neutrophil activation by strains of *H. pylori* isolated from patients with peptic ulcer and chronic gastritis"(chapter 3). During the period between 1995-1996, another 72 patients were recruited into the studies involving "Association of mucosal levels of interleukin-8 and reactive oxygen metabolites and their relationship to toxinigenicity of *H. pylori*"(chapter 5). Some of these patients were also studied for the relationship between mucosal IgA *H. pylori* antibodies and toxinogenicity of *H. pylori*(chapter 6). Patients with duodenal ulcer and with *H. pylori* infection were selected and treated with eradication therapy(chapter 7).

## 2.2 Endoscopy and Biopsies

Olympus GIQ endoscopes were used. During endoscopy, oesophagus, stomach and duodenum were sequentially examined and any abnormalities recorded. Mucosal biopsies were taken using standard size biopsy forceps for histological examination including assessment of inflammation and detection of *H. pylori*, culture, urease testing(CLOtest) and measurement for ROM, IL-8 and mucosal antibodies. All biopsies were placed in appropriate medium and transported to laboratories and processed within two hours.

## 2.3 Histological examination

Mucosal biopsies were fixed in formalin and sent for histological examination. Biopsy specimens were stained and examined by an independent pathologist in a blinded fashion.

### *2.3.1 Assessment of inflammation*

The standard haematoxylin and eosin(H&E) staining method was used to assess mucosal inflammation. The severity of the mucosal inflammation was graded according to degree of infiltration of neutrophils and chronic inflammatory cells(mainly lymphocytes and plasma cells)(Table 1).

Table 1 Histological assessment of inflammation of antral mucosa according to neutrophil and mononuclear cells infiltration.

| Scoring(grade) of inflammation | Type of inflammation | |
|---|---|---|
| | Acute(neutrophils per high power field) | Chronic |
| 0 (normal) | 0 | Few scattered mononuclear cells |
| 1 (mild) | 1-10 | Slight increase in numbers of mononuclear cells |
| 2 (moderate) | 11-20 | Moderate numbers of mononuclear cells |
| 3 (severe) | >20 | Large numbers of mononuclear cells |

### *2.3.2 Detection of H. pylori*

Cresyl fast violet stain was used to detect *H. pylori* and was found to be simple and reliable(140). *H. pylori* density was graded from 0-3 corresponding to absence, rare, moderate or heavy colonisation.

## 2.4 Cultivation of *H. pylori*

### *2.4.1 Transportation of biopsy specimen and culture*

Biopsies were taken at endoscopy and placed in 0.5ml saline and transported to the Bacteriology laboratory and processed within two hours. Each biopsy were weighed and placed in an Eppendorf tube containing phosphate buffered saline(PBS, pH 7.3). The volume of PBS was adjusted to a ratio of one mg biopsy/100 μl of PBS. The content was then homogenised gently in a tissue homogeniser(BDH, Merck Ltd, Dorset, UK). 10 μl homogenate was then diluted with 90 μl of saline, inoculated onto a Columbia blood agar plate and spread evenly over the whole surface of the plate using a sterile loop. The plates were incubated under microaerobic conditions(5-6%$O_2$, 8-10%$CO_2$, 80-85%$N_2$) by using a BBL Campypak (BBL, Cokeysville, Massachusetts, USA) at 37°C. The plates were inspected daily for up to 7 days.

### *2.4.2 Identification of H. pylori*

Colonies likely to be *H. pylori* were Gram stained and examined microscopically for characteristic morphology. Urease activity was tested by inoculating a loopful of the organism into 0.25ml of urea containing medium(200mM urea and 0.07% phenol red in PBS, pH 7.3). A colour change to pink indicated urease positivity. Catalase activity was tested by inoculating the suspected colonies into a drop of 3% $H_2O_2$ (Sigma, UK) on a glass slide.

Positivity was indicated by instant formation of bubbles of oxygen. To test for oxidase activity, a colony was transferred onto the surface of a commercial oxidase strip; a deep blue/black colour change indicated the presence of oxidase.

***2.4.3 Semiquantative estimation of H. pylori density***

Biopsy homogenates were used to measure *H. pylori* density by counting the colony-forming unit(cfu) at an appropriate dilution. The result was expressed as cfu/mg of biopsy.

***2.4.4 Preservation of H. pylori***

A loopful of *H. pylori* was placed into a Eppendorf tube containing 0.5 ml Brucella broth containing 15% foetal calf serum and 15% glycerol and stored at -70°C.

To recover the frozen bacterium, the broth containing the organism was thawed and pippetted onto Columbia blood agar plates. The plates were then incubated microaerobically as described earlier.

***2.4.5 Culture of H. pylori in Brucella broth***

20 ml Brucella broth(Unipath, Basingstoke, UK) containing 5% foetal calf serum(Sigma, Dorset, UK) was inoculated with 5 loopfuls of the organism taken from agar plates. It was then incubated microaerobically at 37°C on a rotary shaker at 100rpm for 48 hours. After 48 h, the broth cultures were examined microscopically for the typical morphology of curved bacilli and the percentage of coccoid formed strains in each culture was recorded. A broth culture without *H. pylori* was also prepared in the same way as a control.

## 2.5 Measurement of urease

***Procedure***

Biopsy urease testing was performed using a commercial CLO-test kit(Delta West, Australia), which contains agar containing urea and pH indicator. The biopsy specimen was placed into the agar medium and a colour change from yellow to pink red was recorded as positive.

The urease activity of *H. pylori* isolates was tested using a urea containing medium as described earlier.

## 2.6 Measurement of *H. pylori* cytotoxin

### *2.6.1 Background*

Based on the finding that some strains of *H. pylori* were cytotoxic(208), broth culture filtrates of the bacterium caused vacuolation of several cultured cell lines such as Vero cells and HeLa cells. Cover et al(203) has established a method using neutral red to quantify the degree of cell vacuolation. Neutral red is taken up by the vacuolated cells and the amount of the neutral red is proportional to the degree of vacuolation present in the cells. Cytotoxic strains have been associated with a more serious pathology(184,210).

### *2.6.2 Procedure*

Brucella broth cultures of *H. pylori* were obtained as described earlier and centrifuged at 4°C at 3000rpm for 15 min. The supernatants were collected and filtered through 0.4μm pore size and then 0.2μm pore size membrane filters(Millipore, France). The culture filtrates were then transferred to dialysis tubing(Sigma, Dorset, UK) capable of retaining molecules with molecular weight greater than 12KD and concentrated 20-fold by reverse dialysis versus polyethylene glycol(Sigma, Dorset, UK) as described by Taha et al(283). Cultured Vero cells(Fig 2) in Dulbecco's modification of Earle's maintainance medium(Gibco BRL,

Paisley, Scotland) were used to measure intracellular vacuolation caused by the cytotoxin according to the methods of Cover et al(192). Each concentrated culture filtrate was diluted from 1:5 to 1:160. a 10 µl sample was added to each well containing Vero cells in 100 µl Earle's medium of a 96-well tissue culture plates(kindly provided by the Regional Virus Laboratory, Ruchill Hospital, Glasgow). It was then incubated in a $CO_2$ incubator(5% $CO_2$) for 24 h. Cell vacuolation was examined both visually under microscope(Fig 3) and spectrophotometrically by following the uptake of neutral red(Fig 4). The maximum dilution of supernatant that produced vacuolation was defined as the titre of cytotoxin. For the assay, uninoculated broth culture filtrate was used as a negative control.

## *2.7* **Detection of cagA gene in *H. pylori***

### *2.7.1 Background*

Immunological studies have shown that a subset of *H. pylori* strains express a protein with a molecular weight between 120 and 130 kDa, and this protein has been found to be associated with duodenal ulceration(91,210). Because of its close association with cytotoxin production, it has been named cytotoxin-associated gene A protein(CagA). The gene encoding the CagA protein has been named cagA.

### 2.7.2 *Reagents*

TE50 buffer: 50 mM tris, 50 mM EDTA, pH 7.8.

TE10 buffer: 10mM Tris-10mM EDTA, pH 7.8.

TE buffer: 10mM Tris- 1mM EDTA, pH 7.4.

Lysozyme: Sigma, UK

Fig 2 Normal Vero cells(magnification × 400)

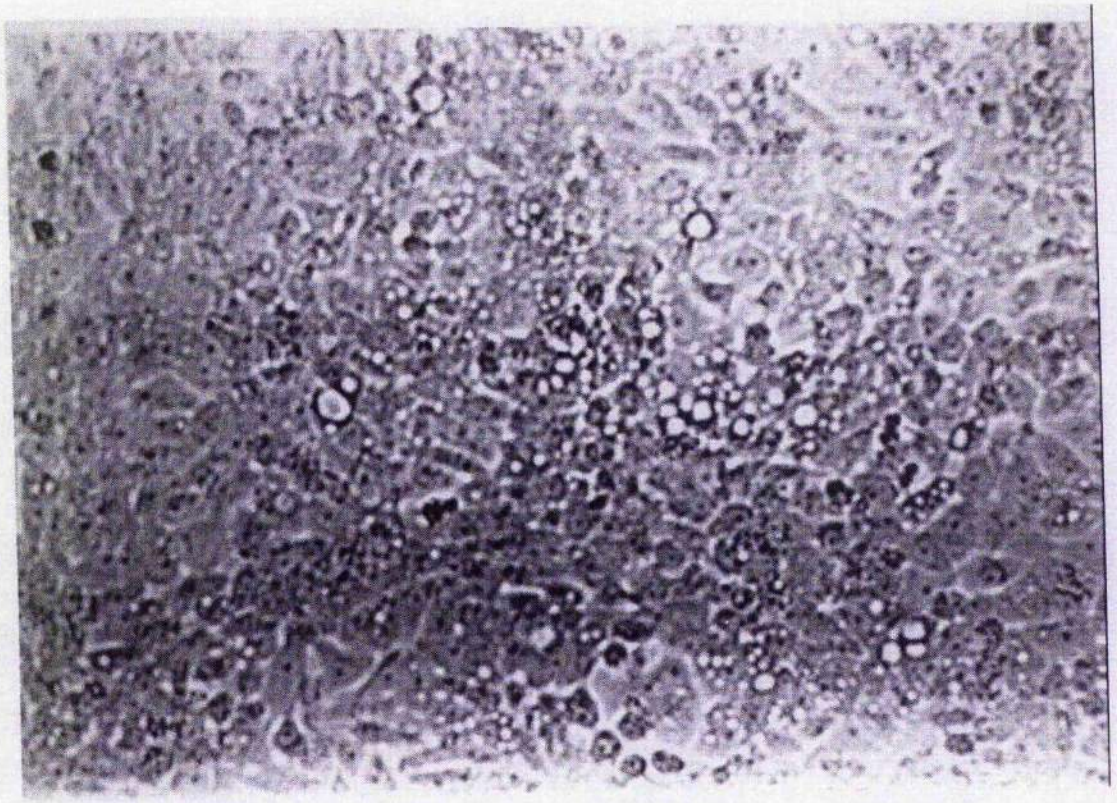

Fig 3 Intracellular vacuolation in Vero cells caused by cytotoxin a positive culture supernatant of *H. pylori*(magnification × 400).

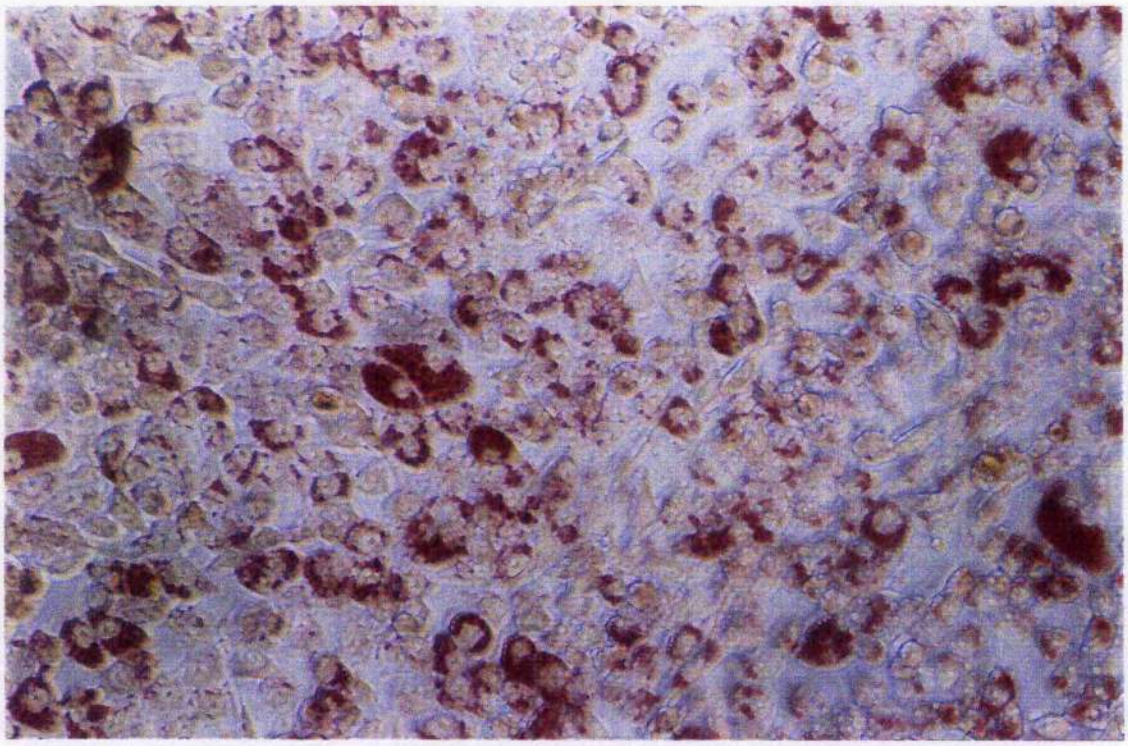

Fig 4 Intracellular vacuolation in Vero cells measured by neutral red uptake assay(magnification×400).

SDS: Sodium dodecyl sulphate, Sigma ,UK

Proteinase K: 10mg/ml, Sigma, UK

Ribonuclease: 10mg/ml, Sigma, UK

TBE buffer: 86 mM Tris, 89 mM boric acid, 1.2 mM EDTA, pH8.2.

Loading buffer: 25%sucrose, 0.068%sodium acetate, 0.1%SDS, 0.005% bromophenal blue.

Primers for cagA PCR: primer 1 5'-AGGAATCTCGCAATTAAGGG-3'

primer 2 5'-TTCTATGCCATTATGACTCCCC-3'

***2.7.3 Procedure***

***Extraction of chromosomal DNA***

strains of *H. pylori* were cultured on Columbia blood agar plates. Colonies were collected for each strain into 5 ml of TE50 buffer. After centrifugation at 3000rpm for 10 min, the pellet was resuspended in TE50 buffer and transferred to Eppendorf tubes. 50μl lysozyme was added(final concentration at 3mg/ml) on ice for 30 min followed by the addition of 25μl of 20% SDS(final concentration 1%) and left on ice for 10min. 50 μl of proteinase K(10mg/ml)(final concentration 1mg/ml) was added and incubated at 56°C in water bath for 2 h. To extract DNA, 500 μl of phenol-chloroform was added and mixed. After centrifugation at 13000rpm for 10min, the upper aqueous layer was transferred to another Eppendorf tube. 500μl of isopropanol was then added and left for 2 h at -20° C. The DNA pellet was resuspended in 0.3 ml TE10 and precipitated by the addition of 100μl of 7.5M ammonium acetate and 600μl of 95% ethanol and left at -20°C overnight. The DNA pellet was obtained by centrifugation, and resuspended in 0.3ml TE10. 10 μl ribonuclease(10mg/ml) was added and

incubated at 37°C for 1 h. DNA was extracted with phenol-chloroform, and precipitated with 0.5ml isopropanol and recovered by centrifugation. The DNA pellet was dissolved in 0.3ml TE10, precipitated by the addition of 100μl ammonium acetate and 0.6ml 95% ethanol. The precipitated DNA was obtained by centrifugation, rehydrated in 60 μl TE buffer and stored at 4°C.

***Polymerase chain reaction(PCR) for cagA gene***

Approximately 20ng of the DNA preparation was used in a PCR mixture of 25μl(Ready To Go, PCR Beads, Pharmacia, UK) containing 1.5units of Taq polymerase, 10mM Tris-HCI, 50mM KCI, 3mM MgCI2, 200μM of each dNTP and stabilizers, including BSA, and 0.2μl of each primer. Primers were selected by Ende et al(284) from published sequences(91).

The reaction conditions were as follows: 5 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 60° C, and 1.5 min at 72° C, followed by 4 min at 72° C and then chilled to 4° C.

***Analysis of the PCR products***

The PCR products were analysed by horizontal agarose(1.5%) gel electrophoresis, by adding 15μl sample with 5μl of loading buffer into each preformed well of the gel. The running conditions were 100 volts for 1 h in an electrophoresis tank(Horizon 58, BRL, Life Technologies, UK) containing TBE buffer. After electrophoresis, the gel was viewed on an ultraviolet box and a picture was taken by a Polaroid camera with a red/orange Kodak filter.

## *2.8* Measurement of phagocytosis by chemiluminescence

### *2.8.1 Background*

Phagocytosis is the process by which bacteria are ingested and killed by white blood cells such as neutrophils and macrophages. During phagocytosis, large amounts of reactive oxygen metabolites, including superoxide anion, hydrogen peroxide and hydroxyl radicals are released.

Luminescence is the emission of light by a non-thermal process. In the case of chemiluminescence, the light is produced by a chemical reaction. Luminol is a synthetic compound which emits light when oxidized by either peroxides or oxygen radicals.

$$Luminol + H_2O_2 \longrightarrow alpha\text{-}aminophthalate + N_2 + H_2O + light\ (425nm)$$

Measurement of the light released during the reaction can be used to detect peroxidase, oxygen radicals or systems producing these substances. The intensity of the light released is proportional to the concentration of reactants and can be monitored by a light detector called luminometer.

The advantages of the luminescence assay over conventional techniques such as colorimetry, spectrophotometry and the counting of radioactive emissions are higher sensitivity and specificity, safer use and rapid measurement.

### *2.8.2 Reagents*

***White Cell Separation Reagents***

Polymorphprep™: Sodium Metrizoate, 13.8%, Dextran 500, 8.0%.(Nycomed, Birmingham, UK).

gel-Hanks solution: Hanks Balanced Salt Solution(HBSS) containing 0.1% gelatin

White cell counting fluid: 2% glacial acetic acid(*v/v*) in distilled water, containing 100µl of 1% methylene blue in 100ml.

Luminol: 5-amino-2,3-dihydro-1,4-phthalazinedine(Sigma, UK).

Stock solution: $10^{-2}$M, working solution $10^{-5}$M.

### *2.8.3 Methods*

***Preparation of bacterial cells***

Fresh cultures of *H. pylori* from Columbia blood agar plates were collected and washed three times with sterile saline. A bacterial suspension was prepared and adjusted to a concentration of approximately $5\times10^7$/ml by diluting the bacterial suspension to an optical density(OD) of 0.45 at 620nm.

***Isolation of Polymorphonuclear leukocytes(PMNs)***

Healthy young adults without a history of peptic ulcer and under no treatment were used as white cell donors. Fresh blood samples(40-60 ml for each experiment) were obtained and placed into in a universal container with 0.2 ml heparin and mixed by inverting gently. PMNs were isolated by using Polymorphprep™ solution and density gradient centrifugation. After centrifugation, the PMN layer was removed. The PMNs were then washed twice in gel-Hanks solution and standardised to contain $1\times10^7$/ml.

***Chemiluminescence assay to measure bacterial phagocytosis***

ROM formation by PMNs in response to stimulation by strains of *H. pylori* was measured by a luminol-dependent CL assay. Nonopsonised strains or strains opsonised with 10% normal human serum(for 30 min) were tested. 50μl of bacterial suspension was added to 100 μl of PMN preparation. Just before the assay, 50 μl of luminol($10^{-5}$ M) was added. The oxidative burst of PMNs was recorded in CPS(count per second) in a Luminometer(Canberra Packard, Caversham, UK). The peak value(CPS) and the time(min) to reach the peak value were recorded for each strain. The assay for each sample was counted at 3-4 min interval for at least 60 min and

each bacterial suspension was tested three times. The average reading for each strain was taken.

## 2.9 Measurement of mucosal reactive oxygen metabolites(ROM) by chemiluminescence

### *2.9.1 Background*

ROM have been implicated in many inflammatory disorders, including those of the gastrointestinal diseases(285). The infiltration of neutrophils and monocytes associated with gastrointestinal mucosal inflammation can provide an important source of ROM via the enzymes of the respiratory burst(NADPH oxidase and myeloperoxidase) and those involved in prostaglandin and leukotriene metabolism(286).

Luminol can react with oxidants such as ROM to form 3-aminophthalate and N-methylacridone. The excited electrons in these compounds revert to their ground state with the emission of light(luminescence), which can be detected by a light detector such as luminometer(287).

### *2.9.2 Procedure*

Each biopsy specimen was bathed in and transported separately in a small volume of phosphate buffered saline(pH 7.3). After the biopsies were weighed, luminol-enhanced CL was performed using a luminometer(BioOrbit, UK), in which the light emission accompanying the generation of ROM is measured and the intensity of light produced expressed as millivolts(mv). Tubes containing one ml of 50 μM luminol were measured for background chemiluminescence. After addition of the biopsy,

sample tubes containing one ml of 50 μM luminol were remeasured for 5 min. The results were expressed as mv/min/mg biopsy after subtraction of the background.

## *2.10* Measurement of mucosal levels of IL-8

### *2.10.1 Background*

IL-8 is a potent chemotactic factor and activator of neutrophils. By using measurements of mRNA and the protein levels of IL-8, it was found that *H. pylori* infected gastric mucosa displayed increased expression of IL-8.

### *2.10.2 Procedure*

***Sample preparation***

Each gastric antral biopsy was immediately washed with phosphate buffered saline(pH 7.3), weighed and placed in an Eppendorf tube containing 0.5ml phosphate buffered saline(pH 7.3). It was then homogenised using a glass grinder(BDH, UK) at 4°C. The resulting suspension was centrifuged at 2000 g for 10 min and the supernatant collected and stored at -70°C before measurement of IL-8.

***Measurement of IL-8 by ELISA***

IL-8 was measured by ELISA(Bio-Whittaker, UK), each assay being performed in duplicate according to the manufacturer's instructions. Briefly, microwell strips coated with monoclonal antibody to human IL-8 were washed before 50 μl of each sample supernatant was added. 50 μl of diluted conjugate was then added to every well including those containing IL-8 standard and incubated for 2 h. After washing, 100 μl substrate solution was added and incubated for 15 min. The enzyme reaction was stopped by the addition of 4N sulphuric acid and the absorbance was measured by an ELISA reader(Labsystems, UK) set at 450 nm. The concentration of IL-8 in each

sample was calculated against a standard curve. The results were expressed as pg/mg biopsy.

## *2.11* Measurement of *H. pylori* antibodies

### *2.11.1 Background*

*H. pylori* infection induces significant local and systemic immune responses. Various serological tests have been developed to detect specific antibodies to the bacterium. IgG isotype has been used widely in serological studies, while IgA is the predominant isotype at the local mucosal level and in gastric juice. Current commercial ELISA kits using partially purified antigens containing urease, provide fairly high sensitivity and specificity(155,156).

### *2.11.2 Methods*

***Biopsy specimen preparation***

Sample used for measurement of mucosal antibodies was the same as the sample preparation for measurement of IL-8 described earlier.

***Measurement of H. pylori antibody by ELISA***

Mucosal *H. pylori* antibodies were measured by using ELISA kits(Sigma, UK) in duplicate according to the manufacturer's instructions. The procedure was as follows. The samples were diluted 1 in 50 in PBS/Tween 20(0.25%) and 100 μl was added to each well. The plates were incubated at room temperature for 40 min and then washed three times in PBS/Tween 20. Diluted(1 to 5000 in PBS/Tween 20) peroxidase conjugated goat anti-human IgA was added to each well(Sigma, UK). The plates were then incubated at room temperature for 30 min. After washing three times, 100 μl of substrate solution(containing DMB and $H_2O_2$) was added and incubated at room temperature for 20 min. The

reaction was stopped by adding 100 μl of 2.5 N sulphuric acid. The optical density(OD) at 450 nm was measured on the Titertek Multiscan plate reader(Flow Laboratories, Irvine, Scotland, UK). Two reference samples(one positive and one negative) were also included in each experiment. Antibody positivity was defined as the OD value greater than 2 SD(standard deviation) of the mean OD of *H. pylori*-ve samples. The antibody titre was expressed as follows: Antibody titre = (sample OD - (mean + 2 SD of negative OD))× 1000.

## 2.12 Eradication therapy of *H. pylori* infection

### *2.12.1 Background*

Studies have confirmed the association between *H. pylori* infection and the development of peptic ulceration and the eradication of the organism leads to the cure of peptic ulcer associated with the infection. Eradication therapy is recommended in patients with peptic ulcer disease with *H. pylori* infection. Current preferred regimes for *H. pylori* eradication include a proton pump inhibitor(such as omeprazole or lansoprazole), and two antibiotics such as metronidazole and clarithromycin.

### *2.12.2 Patients and treatment regimen*

Patients with duodenal ulcer and infected with *H. pylori* were treated with lansoprazole 30 mg daily for four weeks, metronidazole 200 mg three times daily for one week and clarithromycin 250 mg three times daily for one week.

CHAPTER 3

# Association of cytotoxin production by strains of Helicobacter pylori isolated from patients with peptic ulceration and chronic gastritis and neutrophil activation

## 3.1 Introduction

The mechanisms by which *H. pylori* causes gastroduodenal mucosal damage are not well established. Free oxygen radicals have been implicated in a wide spectrum of human diseases(239), including gastrointestinal disorders(32,285) and may play an important role in the development of gastroduodenal inflammation and peptic ulceration(244). A prominent histological feature of infection with *H. pylori* is a dense infiltration of polymorphonuclear cells in the epithelium and the underlying lamina propria, which could provide an important source of free radicals(238). Once activated, the neutrophils release free oxygen radicals which may damage mucosal integrity(288). There is evidence to show that *H. pylori* stimulates gastric antral mucosal reactive oxygen radical production *in vivo*(244).

Several potential virulence factors of *H. pylori* such as cytotoxin and urease may contribute to mucosal damage(190,165). Recent studies suggest that the cytotoxin and the CagA protein are associated with peptic ulceration and chronic active gastric inflammation(189,93).

Surface proteins of *H. pylori* which contain urease have been shown to activate human blood monocytes(175), leading to the secretion of inflammatory cytokines and reactive oxygen intermediates, although the protein(s) involved

was not identified. Another protein with an estimated molecular weight of 150KD has also been associated with neutrophil activation(289).

## 3.2 Aims of study

To investigate whether a relationship exists between cytotoxin-producing strains of *H. pylori* and the generation of free oxygen radicals by neutrophils and to determine whether a relationship exists between neutrophil activation and disease status.

## 3.3 Patients and experimental design

76 patients undergoing endoscopy in Glasgow Royal Infirmary were recruited, consisting of 45 with peptic ulcer(36 duodenal ulcer, 9 gastric ulcer), and 31 with chronic gastritis only. 44 patients were male(age between 20-75, mean 57), and 32 were female(age 24-73, mean 55). At endoscopy, gastric biopsies were taken for culture, CLO test(Delta West Ltd, Australia) and histology.

76 clinical isolates of *H. pylori* isolated from each of the 76 patients were studied; All were positive for urease, oxidase, catalase and showed typical morphology under light microscopy. For cytotoxin measurement and chemiluminescence(CL) assay, the strains were cultured in Brucella broth containing 5% foetal bovine serum at 37° C under microaerophilic conditions as described in chapter 2. After 48 h, the broth cultures were examined microscopically for the typical morphology of curved bacilli and the percentage of coccoid forms in each culture was recorded. The percentage was found to be similar for all the strains in this study, which was 18-20%. The cultures were then centrifuged at 3000rpm for 15min, the bacterial cells collected for CL

assay, and culture supernatants for cytotoxin assay. A broth culture which had not been inoculated with *H. pylori* was also prepared in the same way.

The cytotoxin assay was performed following the method described earlier(chapter 2).

Free oxygen metabolites(ROM) formation by polymorphnuclear neutrophils(PMNs) in response to *H. pylori* was studied by a luminol-dependent CL assay, following the methods described earlier(chapter 2). Peak CL count(as CPS) and the time to reach peak(in minutes) were recorded. Figure 5 shows two representative curves of CL induced by two strains of *H. pylori*.

76 nonopsonised strains and 42 strains opsonised with 10% normal human serum were tested in parallel. Healthy young adults without a history of peptic ulcer disease and under no treatment were used as white cell donors. Sera used for opsonisation were tested for anti-*H. pylori* IgG antibodies by ELISA.

***Statistical methods***

$X^2$ test and Student's t test were used for two group comparisons. A two-way analysis of variance and multiple comparison procedure was used to determine the independent effect of toxinogenicity and the presence/absence of peptic ulcer on the PMN chemiluminescence response induced by strains of *H. pylori*.

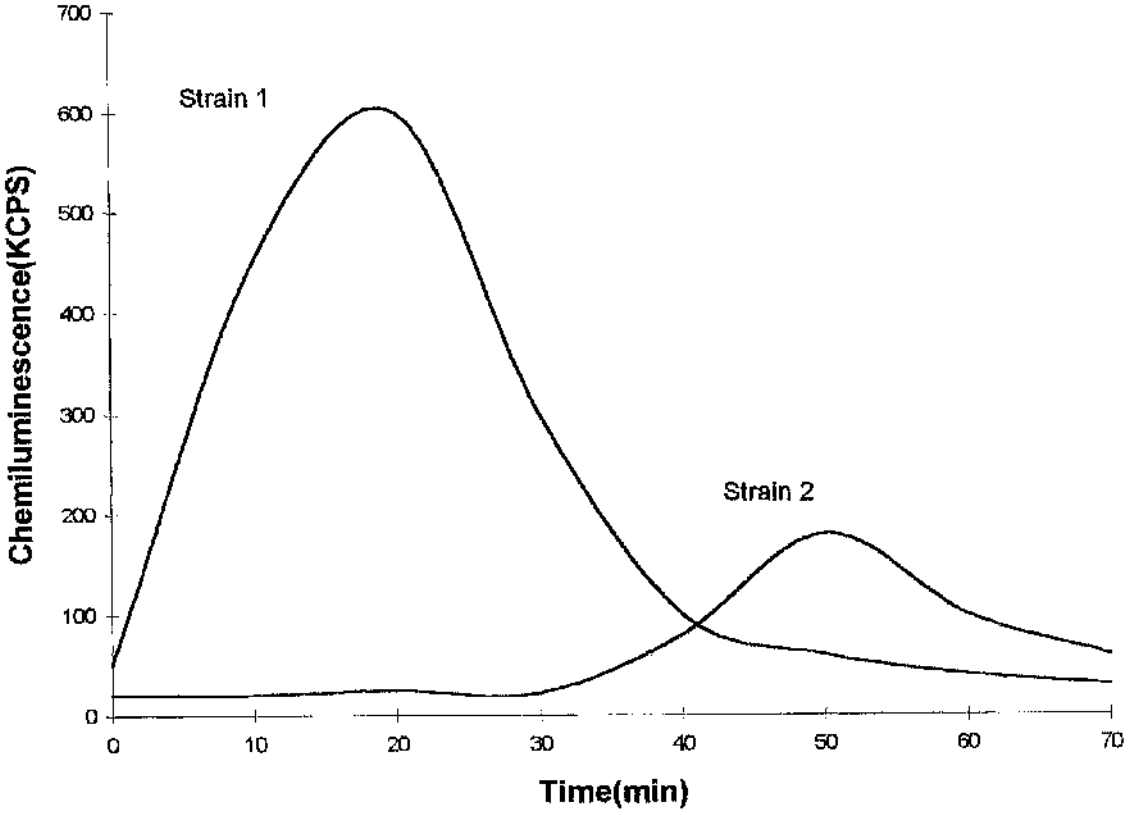


Fig 5 Chemiluminescence(count per second, CPS) induced by two representative strains of H. pylori. Strain 1 induced a stronger and quicker CL response than strain 2.

## 3.4 Results

***Production of cytotoxin***

Concentrated culture supernatants from 39/76(51.3%) of the isolates of *H. pylori* produced measurable cell vacuolation. Among the strains from peptic ulcer patients 30/45(66.7%) induced cell vacuolation versus 9/31(29%) strains from patients with chronic gastritis only($p<0.01$) (Table 2). Some variation in toxinogenicity of the various strains was seen with titres of toxin ranging from 0 to 160.

*Table 2* Cytotoxin production by strains of *H. pylori* isolated from 76 patients with either peptic ulcer or chronic gastritis only.

| Toxinogenicity of strains | Prevalence among patients with Peptic ulcer | Chronic gastritis only |
|---|---|---|
| Tox + | 30/45* | 9/31 |
| Tox - | 15/45 | 22/31 |

* $p<0.01$, compared with patients with chronic gastritis only($X^2$ test).

***Generation of reactive oxygen radicals measured by chemiluminescence***

Figure 6 shows the chemiluminescence response induced by 76 non-opsonised strains of *H. pylori*. It shows that there is strain to strain variation in the ability to induce an oxidative burst in PMNs displayed by the difference in the peak

to induce an oxidative burst in PMNs displayed by the difference in the peak count(KCPS) and the time to reach peak(min). Some produced a stronger and more rapid response than others. The CL pattern for each strain was reproducible. Although there are some small variations in peak height for each strain on each test occasion, the peak time was fairly constant(Table 3).

*Table 3* Chemiluminescence(CL) response induced by two representative strains of *H. pylori* in three experiments.

| Strain number | test | Peak value(Kcps) | Peak time(min) |
|---|---|---|---|
| 5617B | 1 | 534 | 15 |
| | 2 | 490 | 15 |
| | 3 | 458 | 16 |
| | | 494(38.2)* | |
| 7994K | 1 | 118 | 39 |
| | 2 | 90 | 40 |
| | 3 | 71 | 40 |
| | | 93(23.6)* | |

* Mean(standard deviation, SD), Strain 5617B induced a higher and quicker CL response. 7994K induced a weaker and slower one.

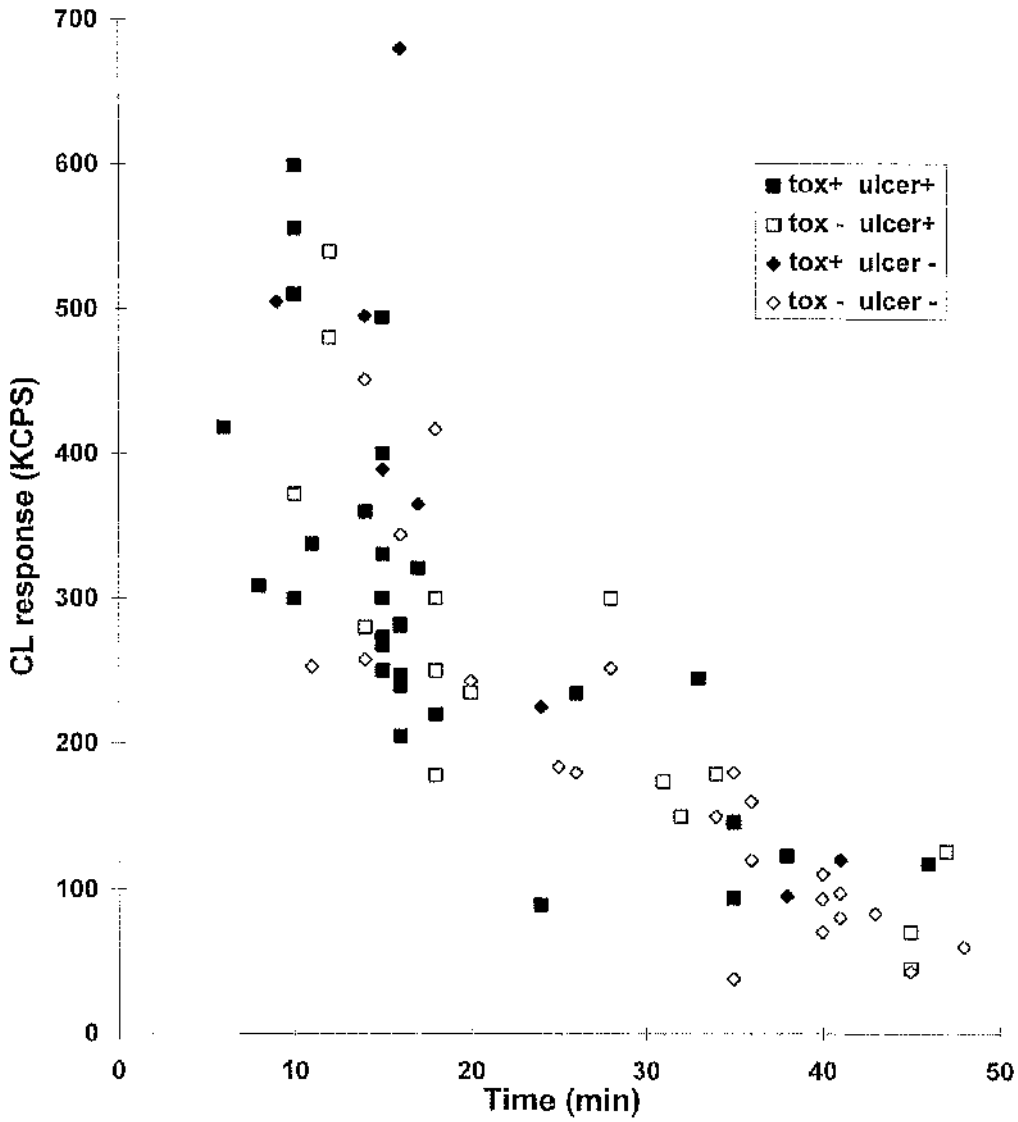


Fig 6 Neutrophil respiratory burst induced by 76 clinical isolates of H. pylori. Results are the mean of three separate experiments and shown as peak count(KCPS) and the time to reach peak(min).

***Reactive oxygen radicals, cytotoxin production and the presence of peptic ulcer disease***

Using a two-way analysis of variance approach, the effect of toxin status and the presence or absence of peptic ulcer on each strain's ability to induce a CL response in terms of peak count and the time to reach peak were investigated. The results are summarised in Table 4 and show that the CL response in terms of peak count induced by toxin positive strains regardless of the presence or absence of peptic ulcer(groups 1 and 2) is higher than that induced by those toxin negative strains from patients without peptic ulcer(group 4)($p<0.05$). It also shows that toxin positive strains(groups 1 and 2) take less time to induce peak CL than those strains of group 4($p<0.05$). These results indicate that the toxinogenicity of *H. pylori* may have a significant effect on the CL response, although there is no significant difference between toxin positive strains(groups 1 and 2) and group 3 which were toxin negative but associated with peptic ulceration($p>0.05$). However, Among the toxin negative strains, those from patients with peptic ulcer failed to show any significant enhancement of the CL response compared to those from patients without peptic ulcer(group 4)($p>0.05$) in terms of peak count and the time to reach peak.

*Table 4* Peak count and time to reach peak in the chemiluminescence response induced by 76 strains of *H. pylori* in relation to the toxinogenicity and the presence or absence of peptic ulcer disease.

| Group | Characteristic | No | Mean(SD) | |
|---|---|---|---|---|
| | | | Peak count(Kcps) | Time(min) |
| 1 | Tox+ Ulcer+ | 30 | 295.1(128.1)* | 18.8(9.86)$ |
| 2 | Tox+ Ulcer - | 9 | 346.2(193.9)# | 21.1(11.2)ø |
| 3 | Tox - Ulcer+ | 15 | 245.3(139.8) | 25.6(12.8) |
| 4 | Tox - Ulcer - | 22 | 175.7(116.7) | 31.1(11.3) |

*$p < 0.05$ with a 95% CI(38.6, 200.6) for difference in means between group 1 and 4. #$p<0.05$, 95% CI(5.7, 335.3) between 2 and 4. $$p<0.05$, 95% CI(-19.6, -5.2) between 1 and 4. ø$p<0.05$, 95%CI(-19.2, -0.9) between 2 and 4. (Two-way analysis of variance and multiple comparison).

When opsonised with 10% human serum, most strains of *H. pylori* resulted in a stronger and more rapid CL response than when non-opsonised. A few(7/42) strains which induced a high CL response when non-opsonised gave a slightly decreased peak count(mean decrease:65.4±36.5), while the time to reach peak was relatively unchanged. As can be seen from Table 5, there is no significant difference between the four groups of strains($p>0.05$), indicating that the toxin status and the presence/absence of an ulcer do not affect the CL response induced by opsonised strains. The presence of *H. pylori* antibody in any sera used for opsonisation did not affect the results.

***Table 5*** Peak count and the time to reach peak in the chemiluminescence response induced by 42 opsonised strains of *H. pylori* in relation to the toxinogenicity and the presence or absence of peptic ulcer.

| Group | Characteristic | No | Mean(SD) | |
|---|---|---|---|---|
| | | | Peak count(Kcps) | Time(min) |
| 1 | Tox+ Ulcer+ | 15 | 522.1(165.7) | 13.4(3.7) |
| 2 | Tox+ Ulcer - | 7 | 504.3(168.4) | 13.4(3.6) |
| 3 | Tox - Ulcer+ | 10 | 472.5(128.4) | 13.5(3.8) |
| 4 | Tox - Ulcer - | 10 | 440.2(159.6) | 14.5(4.7) |

$p>0.05$ , Two-way analysis of variance.

## 3.5 Discussion and Conclusion

From the study, 66.7% nonopsonised strains of *H. pylori* from peptic ulcer patients induced cell vacuolation, which is significantly higher than those from patients without proven peptic ulcer(29%). This supports a previous report that the cytotoxin-producing strains were more frequently found in patients with peptic ulcer(189). Using a two way analysis of variance approach, the effect of toxinogenicity and the presence/absence of peptic ulcer on the oxidative burst induced by non-opsonised strains of *H. pylori* was investigated. It was found that cytotoxin-producing strains have an increased CL response in terms of higher peak count and shorter time to reach peak than those toxin negative strains from patients without peptic ulcer. These results support a possible link between the neutrophil respiratory burst and the toxinogenicity of strains of *H. pylori*. The association of certain strains with peptic ulcer disease *per se* did not influence significantly the CL response of human PMN. However, this finding does not exclude a possible relation between the neutrophil respiratory burst and peptic ulceration, since most strains from peptic ulcer patients(66.7% in this study) are cytotoxin-producing. Also we cannot rule out the possibility that some chronic gastritis patients infected with a cytotoxin-producing strain might have had an ulcer in the past or may develop one in the future.

Most opsonised strains of *H. pylori* gave an increased CL response in our study and the serum antibody to *H. pylori* showed no effect on the CL response. This is consistent with previous reports(290,291) which suggested that serum complement rather than serum antibody is the main opsonin in this respect. However, it is not certain whether complement-mediated opsonisation occurs

within the gastric mucosa during *H. pylori* infection. A few opsonised strains gave a decreased CL response as compared to the same strains non-opsonised. The reason for this is not known; some serum proteins may act as electron scavengers(292).

Other investigations have shown that there are some differences between strains of *H. pylori* in their ability to produce cytotoxin and CagA protein and to cause peptic ulcer disease(210). Although host factors play a very important role in the genesis of peptic ulcer, there is increasing evidence from molecular biology and immunology for the division of *H. pylori* strains into at least two major groups–one producing the CagA protein and cytotoxin(Type I) and one that does not produce either of these antigens(Type II)(215). This study showed there is strain to strain variation in the ability to produce cytotoxin, and to induce an oxidative burst in PMNs.

Previous studies have shown that CagA/cytotoxin positive strains of *H. pylori* are associated with the infiltration of neutrophils within the gastric mucosa(192,210) and also related to an increased expression of interleukin-8, which is a novel cytokine capable of activating neutrophils. Based on these results and the findings in this study which indicate a positive association between the neutrophil respiratory burst and the cytotoxicity of *H. pylori* which is also associated with peptic ulceration, it seems, therefore, that cytotoxin/CagA positive strains may induce a more aggressive inflammatory response through secretion of toxic component(s), production of cytokines by gastric mucosal cells, recruitment of neutrophils, release of free oxygen

radicals, and subsequently cause tissue damage, which may lead to peptic ulceration.

There were some strains that did not produce cytotoxin but which could induce a high CL response, raising the possibility that some *H. pylori* product(s) other than cytotoxin may be important in this respect. So far the nature of the component(s) of *H. pylori* which activates neutrophils is uncertain. Several reports have suggested that the activity is mainly caused by a protein(s), although some non-protein molecules such as lipopolysaccharides(LPS) may also be implicated(175,176). There is a possibility that the gene encoding for the protein which activates PMNs is more strongly expressed on the so-called Type I strains that produce cytotoxin and CagA protein.

In conclusion, cytotoxin-producing strains of *H. pylori* appears to be correlated with increased free oxygen radical formation by neutrophils and peptic ulceration. The ability of some *H. pylori* strains to produce cytotoxin and to induce an oxidative burst of neutrophils may be important in the pathogenesis of peptic ulceration.

Chapter 4

# Neutrophil activation by soluble proteins of *Helicobacter pylori*

## 4.1 Introduction

In vitro and *in vivo* studies have suggested H . pylori induces a neutrophil oxidative burst and a subsequent release of reactive oxygen metabolites(235-237,244,245). However, the component(s) of *H. pylori* responsible for the activation of neutrophils is still inconclusive. It has been suggested that some bacterial components of *H. pylori* have chemotactic and/or activating property for leukocytes, including a neutrophil-activating peptide(NAP) and lipopolysaccharide(LPS)(289,293). Previous studies have suggested that cytotoxic strains may induce an enhanced neutrophil oxidative burst(294,295), whereas it is not known whether the cytotoxin *per se* of *H. pylori* can activate neutrophils.

## 4.2 Aims

To investigate the possible component(s) of *H. pylori* culture supernatant which induces LMN chemiluminescence response and to see whether the cytotoxin is involved.

## 4.3 Experimental design

A culture supernatant from a strain of *H. pylori* which stimulated a strong CL response and produced cytotoxin was subjected to various forms of column chromatography. Eluted fractions were tested for cytotoxin, urease activity and ability to induce CL in PMN. The methods are described below —

The cytotoxin producing strain was cultured microaerobically in Brucella broth containing 0.5% charcoal(untreated, granular 8-20 mesh, Sigma, UK). After 48 h, the broth culture was centrifuged at 3000 g for 30 min. The supernatant were than filtered through 0.4μm and then 0.2μm filters(Millipore, France). The culture filtrates were then concentrated 20-fold by reverse dialysis versus polyethylene glycol as described previously(Chapter 2).

***Column Chromotography***

2 ml of concentrated culture supernatant was applied to a Sephadex G-200 column with buffer containing 60 mM Tris and 0.1 M NaCl(pH 7.7) at a flow rate of 0.2ml/min. The eluted proteins were monitored by UV absorbance at 280nm. Individual fractions showing cytotoxin activity were subsequently applied to a Hi-load Q column(Pharmacia) with 20 mM Tris(pH 7.7), and the proteins were eluted with 20 mM Tris containing a linear gradient of 0-1M NaCl over 100 ml. The chromatograms are shown in Figures 7 and 8. The fractions were tested for cytotoxin and urease activity as well as for their ability to activate neutrophils.

***Measuring neutrophil activation by chemiluminescence***

Fractions were tested for neutrophil activating property by chemiluminescence(CL) using a luminometer(Bio-orbit). The assay was performed by adding 100 ul of each protein fraction to 100 ul of PMN($10^7$/ml) and 100 ul of luminol($10^{-5}$M). CL was expressed as mv and the peak(mv) and peak time(min) were recorded. The effect of proteinase K and heat treatment on the activity of the protein fractions were tested either by treatment of the fractions with 2mg/ml proteinase K or by heating for 20 min at 100°C in a water bath. Each test was performed three times.

## 4.4 Results

Gel chromatography

Fig 7 shows that Sephadex G-200 gel chromatography yielded two protein peaks. Fractions of protein peak 1 were shown to be urease positive and cytotoxin positive. They were also shown to induce a neutrophil oxidative burst. These fractions was then purified further by applying to an ion exchange column. As can seen from Fig 8, three protein peaks(a, b, c) were separated following elution with a gradient concentrations of NaCl from 0-1M. Fractions from peak b showed both cytotoxin and urease activity.

***Neutrophil activation***

Fractions from protein peaks a, b and c were tested for neutrophil activating activity(Fig 9). Protein peaks a and c induced a rapid CL response, whereas protein peak b containing cytotoxin and urease only showed only a slow and in-significant CL response.

There was a dose-dependent CL response in terms of the ability of protein peaks (a) and (c ) to induce an oxidative burst(Table 6). After the treatment of proteinase K(2mg/ml) for 1 h, the neutrophil activating activities of protein peaks (a) and (c ) were significantly decreased. However, heat treatment did not reduce the activity.

Table 6 Chemiluminescence(CL) induced by protein peaks (a) and (c ) at different concentrations and the effect of proteinase K and heat treatment on the CL response.

| Protein | Peak (mv) | Peak time (min) | Mean (mv) |
|---|---|---|---|
| Protein (a) 10 ug/ml | 5.0 | 20 | 4.42 |
| 5 ug/ml | 4.2 | 21 | 2.71 |
| 2.5 ug/ml | 3.1 | 23 | 2.21 |
| 10 ug/ml+proteinase K | 1.7 | 28 | 1.60 |
| 10 ug/ml, 100°C ×20 min | 5.3 | 20 | 4.51 |
| Protein (c) 10 ug/ml | 3.2 | 21 | 2.0 |
| 5 ug/ml | 2.6 | 23 | 1.7 |
| 2.5 ug/ml | 2.2 | 26 | 1.6 |
| 10ug/ml+proteinase K | 1.4 | - | 1.38 |
| 10ug/ml, 100°C ×20 min | 4.1 | 20 | 2.4 |

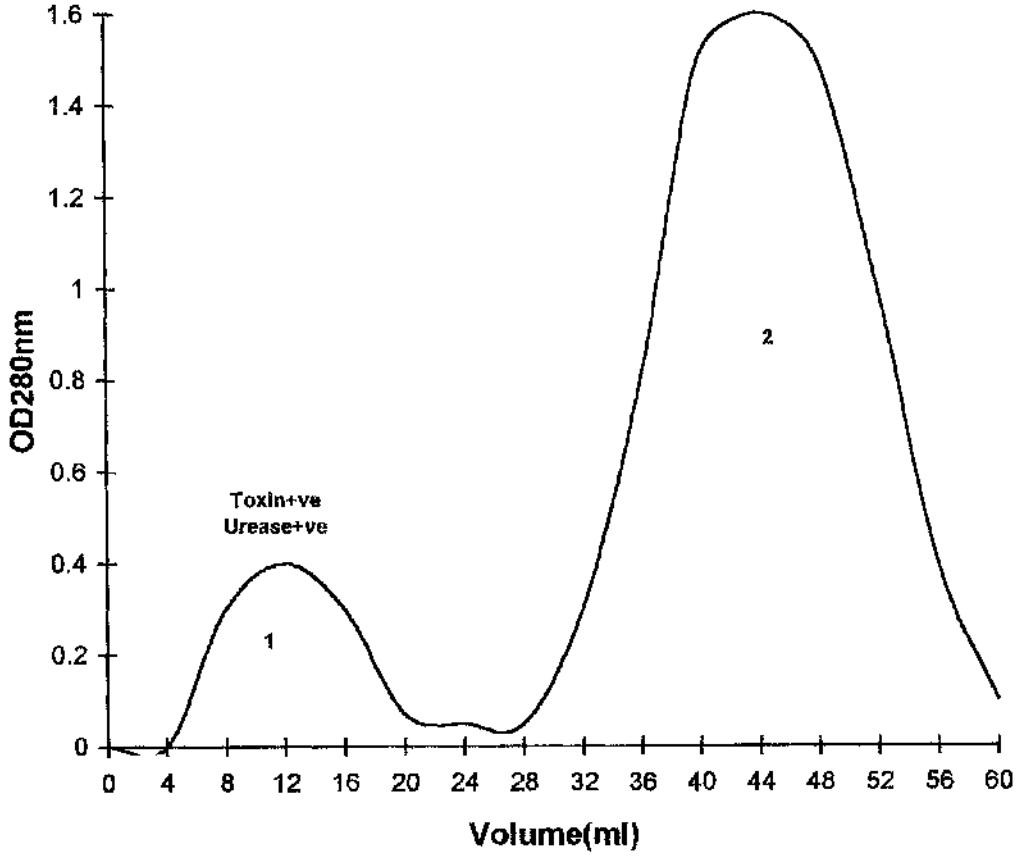


Fig 7 Sephades G-200 Gel chromatography of concentrated culture supernatant of H. pylori. Peak 1 was shown to be cytotoxin positive and urease postive. Horizontal axis shows eluting volume. Vertical axis show optical densities of fractions at 280nm.

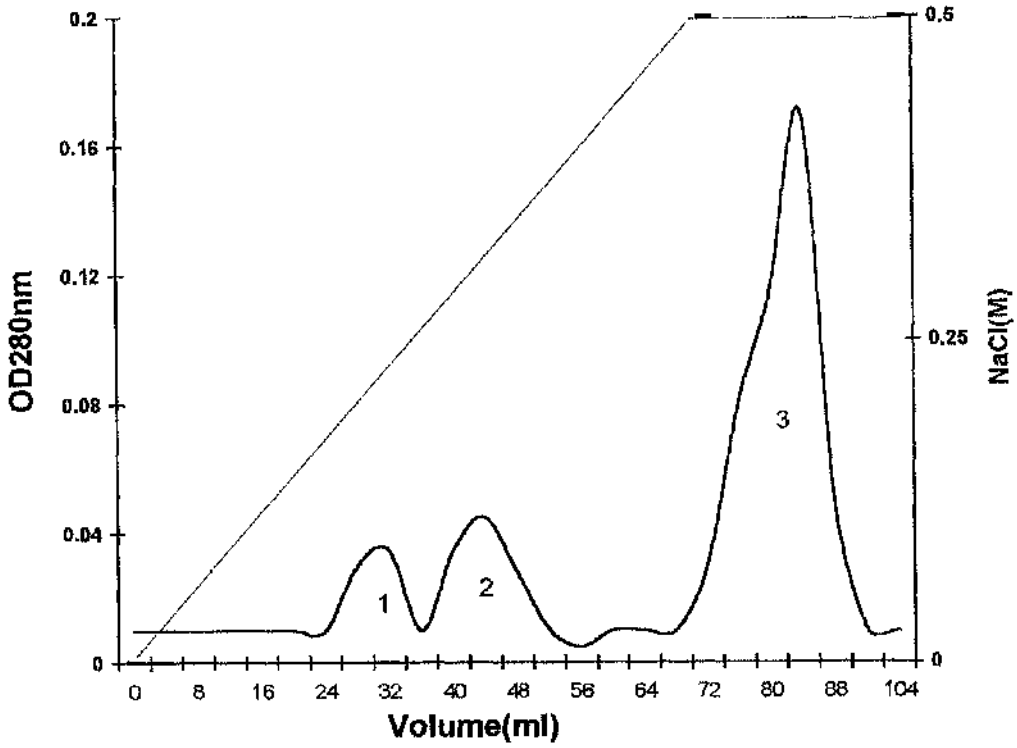


Fig 8 Ion exchange chromatography(Hi-load Q) of protein fractions from gel chromatography. Left axis shows optical densities of fractions. Right axis shows gradient concentration of NaCl(M).

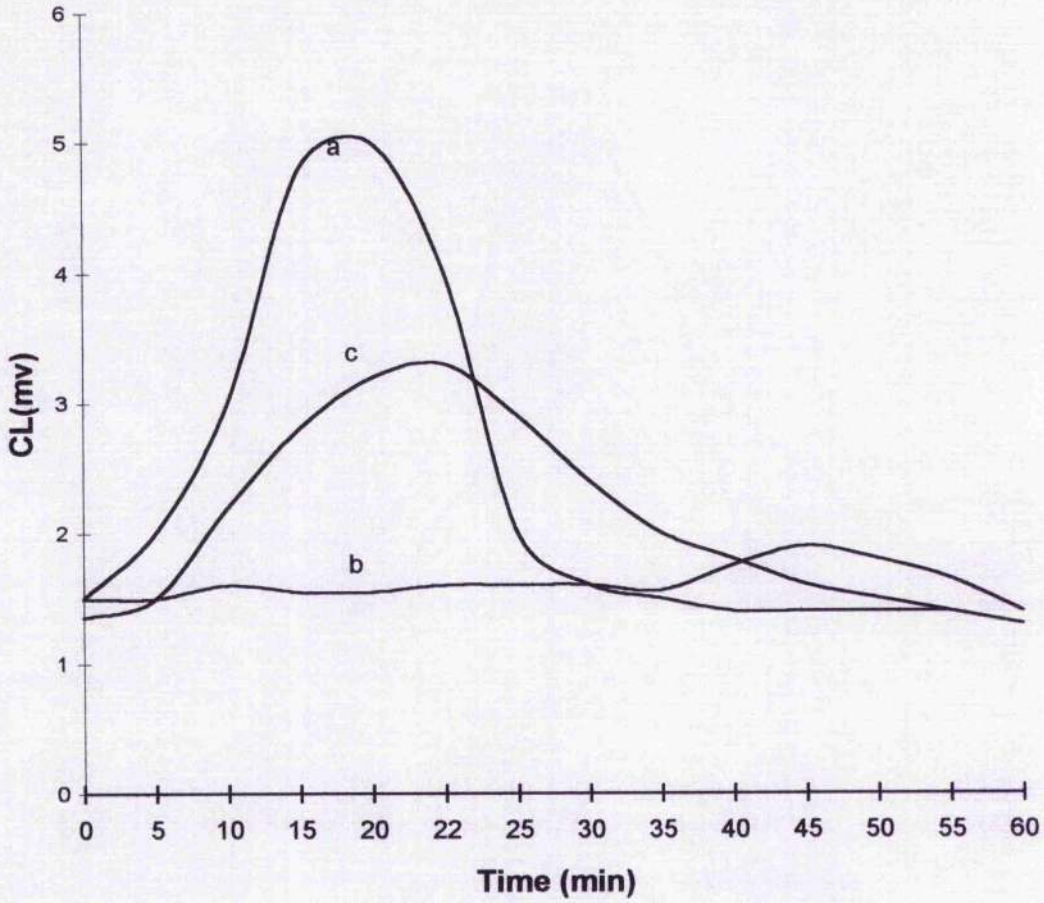


Fig 9 Chemiluminescence(CL) induced by protein peaks purified from ion exchange chromatogrphy. a: CL induced by protein (a). b: CL induced by protein (b). c: induced by protein (c).

## 4.5 Discussion

It was found in this study that soluble products of *H. pylori* could activate neutrophils to release reactive oxygen metabolites. Chromatography showed that there were two protein peaks capable of inducing the CL response, suggesting that more than one product of *H. pylori* was capable of activating neutrophils. The results also showed that the neutrophil activating activity was destroyed or nearly abolished by incubation with proteinase K, suggesting the active components might be polypeptides. However, it is unclear why heat treatment did not abolish the activity as might be expected of a protein.

Previous studies in vitro suggested cytotoxin-producing strains are more likely to induce a stronger neutrophil oxidative burst(294,295). The results in this study have shown that cytotoxin containing fractions from chromatography did not induce a significant CL response suggesting that the cytotoxin *per se* is not the active component. Urease also is unlikely to play a significant role in neutrophil activation, because urease containing fractions failed to induce a significant CL response. However, urease has been shown by others(175) to activate monocytes/macrophages.

Evans et al(289) described a protein with a molecular mass of 150 Kd, which has a significant neutrophil-activating property, namely *H. pylori* neutrophil-activating peptide(HP-NAP). The napA gene encoding the protein was characterised and was shown to be present in all strains of *H. pylori* tested, but it appeared that there is strain to strain variation in the expression of the HP-NAP in vitro(289). It is possible that some of the protein fractions tested in this study contained the NAP protein.

In conclusion, *H. pylori* may secrete several soluble products, possibly proteins, capable of activating neutrophils. The neutrophil activating activity seems to be independent of cytotoxin and urease activity. Further studies are needed to characterise the proteins responsible and to elucidate their clinical importance in *H. pylori*-induced gastroduodenal disease.

CHAPTER 5

# Association of antral mucosal levels of interleukin-8 and reactive oxygen metabolites, and their relationship to toxinogenicity of *Helicobacter pylori*

## 5.1 Introduction

*Helicobacter pylori*(*H. pylori*) infection has now been considered to be the main cause for chronic gastritis and is closely associated with the development of peptic ulcer disease(1-4), although the mechanisms by which *H. pylori* induces mucosal inflammation and peptic ulceration are still not well understood. Only some patients with *H. pylori* infection developed serious pathology such as peptic ulcer disease. Differences in bacterial virulence factors among strains of *H. pylori* and in individual host response to the bacterium are considered to be important in explaining the different clinical outcome(32-34). Currently, two bacterial virulence factors of *H. pylori*, CagA protein(encoded by cagA gene) and the vacuolating cytotoxin(VacA, encoded by vacA), have been suggested to be more frequently expressed in strains isolated from peptic ulcer patients than in those with chronic gastritis only(210,189). Inflammatory cell infiltration including neutrophils and mononuclear cells is characteristic of the gastric mucosal pathology associated with *H. pylori* infection. Recent studies have shown that *H. pylori* can activate neutrophils in vitro(235-237), implicating that neutrophil activation may be important in *H. pylori*-induced mucosal inflammation. Neutrophil activation is an important source of reactive oxygen metabolites(ROM) which are known to cause tissue damage(238,288). Studies in vitro have shown that *H. pylori* infection is

associated with a neutrophil oxidative burst(176,296). *H. pylori* infection has also been shown to be associated with an *in vivo* mucosal production of ROM and higher antral colonisation levels of *H. pylori* are associated with increased production of ROM(244,297).

The intensity of the inflammatory cell infiltrate has been correlated with the severity of mucosal injury(297,298). Interleukin 8(IL-8), a chemokine which attracts and activates phagocytes, especially neutrophils, has been shown to be important in leukocyte-mediated tissue injury(259). Recent studies have shown that gastric mucosal IL-8 is significantly increased in *H. pylori* infected patients, suggesting an important role of IL-8 mediated leukocyte trafficking in *H. pylori* induced inflammation(37,259).

However, there are considerable variations among the infected patients in the mucosal expression of IL-8 and the mucosal production of ROM(37,261). Recent studies in vitro have suggested that IL-8 production is enhanced by cytotoxic/cagA positive strains of *H. pylori* and cagA is considered to be a marker for IL-8 induction(214,269-271). In vitro studies also suggested that cytotoxin-producing strains may induce a stronger oxidative burst in neutrophils(294,295).Currently there are limited data on the association between the different types of *H. pylori* strains and the *in vivo* mucosal production of IL-8 and ROM.

## 5.2 Aims of Study

To investigate (1) whether there is any association between antral mucosal production of IL-8 and ROM; and (2) what their relationship is to toxinogenicity of *H. pylori*; and (3) what their relationship is to gastric antral inflammation.

## 5.3 Patients and experimental Design

Patients referred for endoscopy in the Department of Gastroenterology in Glasgow Royal Infirmary were recruited prospectively during the period between 1994-1996. Patients were excluded if they had previous gastric surgery, had previous *H. pylori* eradication therapy, or were on antibiotics or non-steroidal anti-inflammatory drugs. In total, 72 patients aged between 23 and 78 years(median 41) were enrolled. These included 36 patients with peptic ulcer(32 duodenal ulcer, 4 gastric ulcer) and 36 with non-ulcer dyspepsia. Patients had been advised to stop any anti-acid therapy or ulcer-healing drugs at least two weeks prior to endoscopy.

During endoscopy, gastric antral biopsies were taken using the same size forceps from similar topographical sites at each endoscopy for histology, *H. pylori* culture, CLO test, measurement of tissue levels of IL-8 and chemiluminescence(CL). Histological identification of *H. pylori* in sections stained with H&E and cresyl violet if necessary was assessed by an independent pathologist in a blinded fashion and mucosal inflammation was also scored according to the degree of infiltration of neutrophils and mononuclear cells as described earlier(Table 1). Semiquantitative assessment of colonisation density of *H. pylori* was performed by counting the number of colony forming units(cfu) per mg tissue on culture plates. Cytotoxin production was tested by detection of intracellular vacuolation using cultured Vero cells. The cagA gene expression of *H. pylori* strains was determined by PCR. The PCR products were analysed by horizontal agarose(1.5%) gel electrophoresis(Fig 10). Antral mucosal levels of IL-8 were measured by ELISA. Mucosal production of ROM was measured by luminol-

1 2 3 4 5 6 7 8 9 10 11 12 13

Fig 10 Expression of cagA gene in 11 strains of *H. pylori* analysed by PCR and agarose gel electrophoresis(1.6%). Lane 1 and 13: DNA ladder(123bp). Lane 2: reference strain (NCTC 11637). Lane 3, 4, 6, 8, 9, 10, 11: cagA+ve clinical isolates. Lane 5,7 and 12: cagA-ve clinical isolates. Estimated PCR product: 750 bp.

enhanced chemiluminesence assay. Detailed methods were described in chapter 2.

***Reproducibility of measurement of IL-8 and ROR***

The reproducibility of the two tests were assessed by taking more than one specimen from the same site in each patient. The median(interquartile) percentage disagreements between biopsies from each patient were 8.1%(0.8 to 14.1%)(n=22) for IL-8 and 13.2%(4.1 to 19.5%)(n=32) for CL assay. These variances were relatively small compared to the differences found between patients.

***Statistical analysis***

The Kruskal-Wallis test was used for multiple group comparisons and the Mann-Whitney U test for two-group comparisons. Pearson's correlation analysis was applied to the association between IL-8 levels and ROR production. The relationships between the microscopic inflammation score and IL-8 concentration and ROR production were assessed by Spearman's rank correlation. P values <0.05 were considered to be statistically significant.

## 5.4 Results

***Correlation between detection methods for H. pylori in antral mucosa***

There is a good correlation between the results of *H. pylori* culture, CLO test and histological identification of *H. pylori*. The positivities of the three tests were similar in the biopsies examined; *H. pylori* was detected by all three tests in all 36 peptic ulcer patients(100%) and in 16 of 36(44.4%) of those with NUD.

***Detection of cagA gene and cytotoxin(VacA) in H. pylori strains***

Among the 52 strains of *H. pylori* isolated, 35 expressed cagA gene(cagA+ve), and 30 produced vacuolating cytotoxin(VacA+ve). Of the 35 cagA+ve strains of *H. pylori*, 25(71%) were cytotoxin positive. In those 30 VacA+ve strains, 25(83.3%) were cagA+ve. Correlation between clinical findings, cagA gene expression and cytotoxin-producing status of *H. pylori* strains is summarised in Table 7. 29 of 36(80.6%) strains of *H. pylori* isolated from peptic ulcer patients were cagA positive compared to 6 of 16(37.5%) strains from patients with non-ulcer dyspepsia(p=0.005). 24 of 36(66.7%) strains from peptic ulcer patients produced cytotoxin compared to 6 of 16(37.5%) strains from patients with NUD(p=0.045).

Table 7 Expression of cytotoxin(VacA) and possession of cagA gene of strains of *H. pylori* in patients with peptic ulcer and non-ulcer dyspepsia.

| Patient group | Characteristics of strains | No of patients | | Total |
|---|---|---|---|---|
| | | PU | NUD | |
| 1 | VacA+ cagA+ | 20 | 5 | 25 |
| 2 | VacA- cagA+ | 9 | 1 | 10 |
| 3 | VacA+ cagA- | 4 | 1 | 5 |
| 4 | VacA- cagA- | 3 | 9 | 12 |

There was a good correlation between *H. pylori* colonisation density measured by viable cell counts in culture and that assessed by the histological detection of the organism in biopsies taken from the same area(Fig 11 r=0.58, n=44, p<0.01).

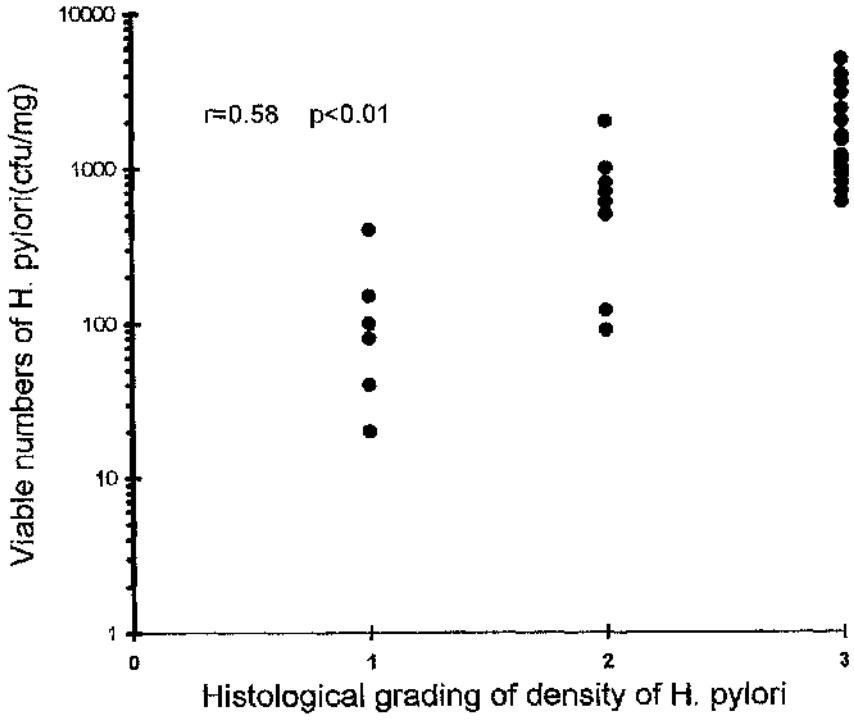


Fig 11 Correlation between H. pylori density assessed by viable count(cfu) in culture and that assessed by histological grading of density.

***Effect of H. pylori infection on gastric mucosal production of IL-8***

Mucosal Levels of IL-8 were significantly increased in samples of antral mucosa colonised with *H. pylori* compared to those without *H. pylori*, regardless of the presence or absence of peptic ulcer($p<0.05$). The levels of IL-8 in those with peptic ulcer was slightly higher than that in those with NUD and with *H. pylori* infection($p=0.034$)(Table 8). No independent effect of patients' age, sex, smoking habit and alcohol consumption was found on mucosal levels of IL-8(Table 9).

Table 8 Antral production of IL-8(pg/mg) in patients with peptic ulcer and non-ulcer dyspepsia(NUD) with or without *H. pylori* infection.

| Patient group | No | Median(interquartile) | |
|---|---|---|---|
| | | IL-8 (*pg/mg*) | ROM(*mv/min/mg*) |
| Peptic ulcer | 36 | 1.50(0.88-2.47)** | 517(45, 730)$^{\#\#}$ |
| Non-ulcer dyspepsia | | | |
| with *H. pylori* | 16 | 0.80(0.45-1.55)* | 143(9, 119)$^{\#}$ |
| without *H. pylori* | 20 | 0.20(0.00, 0.70) | 0(0, 3) |

*p=0.02 compared to NUD patients without *H. pylori*; **p=0.034 compared to NUD patients with *H. pylori*. $^{\#}$p<0.001 compared to NUD patients without *H. pylori*. $^{\#\#}$ p=0.03 compared to NUD patients with *H. pylori*.

***Effect of H. pylori infection on mucosal production of ROM***

Patients infected with *H. pylori* showed significantly higher levels of ROM production(median(interquartile)mv/min/mg) than patients without infection regardless of the presence or absence of peptic ulcer($p<0.001$). The mucosal levels of ROM in peptic ulcer patients were relatively higher compared to those patients with NUD($p=0.031$)(Table 8). A good correlation was found between

antral *H. pylori* density and mucosal ROM release(r=0.68, p<0.001, Fig 12). No correlation was found between mucosal ROM and other factors including patient's age, sex, smoking habit and alcohol consumption(Table 9).

Table 9 Demographic data of patients' sex, age, smoking habit and alcohol consumption and mucosal production of IL-8 and ROM

| Patient | No | Median(IQ range) levels of | | |
|---|---|---|---|---|
| | | IL-8(pg/mg) | ROM(mv/min/mg) | p value |
| Sex | | | | |
| Male | 35 | 0.91(0.44, 1.50) | 36(1, 204) | NS |
| Female | 37 | 0.70(0.30, 1.65) | 30(0, 196) | |
| Age | | | | |
| 20-30 | 13 | 0.77(0.25, 3.30) | 29(1, 805) | |
| 31-40 | 19 | 0.78(0.50, 1.65) | 75(4, 380) | |
| 41-50 | 12 | 0.40(0.29, 1.52) | 56(0, 192) | NS |
| 51-60 | 16 | 0.95(0.40, 1.65) | 20(0, 106) | |
| ≥61 | 12 | 0.70(0.50, 1.00) | 30(0, 120) | |
| Smoking | | | | |
| No | 21 | 0.78(0.35, 2.36) | 48(0, 653) | |
| Moderate | 34 | 0.70(0.25, 1.30) | 16(0, 136) | NS |
| Heavy | 17 | 0.94(0.43, 1.52) | 40(16, 196) | |
| Alcohol | | | | |
| No | 30 | 0.60(0.40, 2.00) | 30(5, 340) | |
| Moderate | 35 | 0.90(0.37, 1.61) | 63(0, 242) | NS |
| Heavy | 7 | 0.83(0.49, 1.00) | 10(0, 90) | |

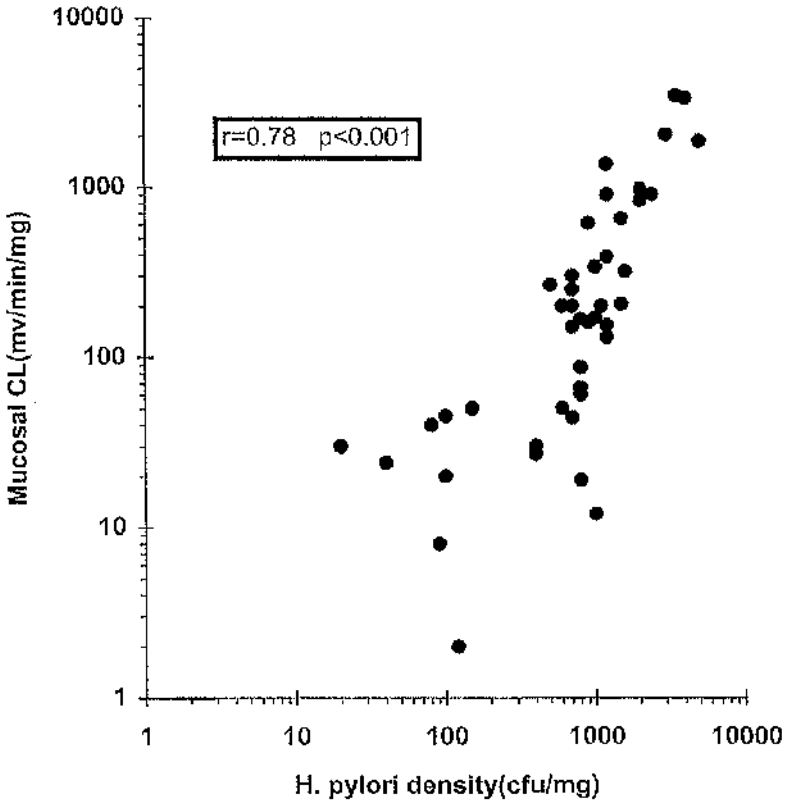


Fig 12 Correlation between H. pylori colonisation density in antrum and mucosal production of ROM measured by chemiluminescnece.

***Relationship between mucosal levels of IL-8 and the mucosal production of ROM***

The relationship between mucosal levels of IL-8 and mucosal production of ROM measured by the chemiluminesence assay was assessed by the Pearson's correlation analysis. There was a positive correlation between antral mucosal concentration of IL-8 and the mucosal production of ROM ($r=0.61$, $p<0.01$)(Fig 13).

***Relationship between mucosal levels of IL-8 and presence of cagA gene and expression of VacA cytotoxin***

Fig 14 and Table 10 show the correlation between antral mucosal protein levels of IL-8 and the presence of cagA gene in the isolates and VacA cytotoxin-producing status of strains of *H. pylori*. Median(interquartile) levels(pg/mg) of IL-8 (1.80(0.97, 2.99)) in patients infected with cagA+ve/VacA+ve strains were significantly higher than that in those with cagA-ve/VacA-ve strains(0.50(0.35, 1.20)) and in those without *H. pylori* infection(0.20(0.00, 0.70)). There was no significant difference in mucosal levels of IL-8 between those patients infected with cagA+ve/VacA-ve strains(0.90(0.63, 2.10)) and those infected with cagA-ve/VacA- strains, although the former group showed higher mucosal IL-8 than patients without *H. pylori* infection. The mucosal IL-8 in patients infected with cagA-ve/VacA+ve strains(0.6(0.28, 1.75)), did not show any significant increase as compared to those infected with cagA-ve/VacA-ve strains or those without *H. pylori* infection.

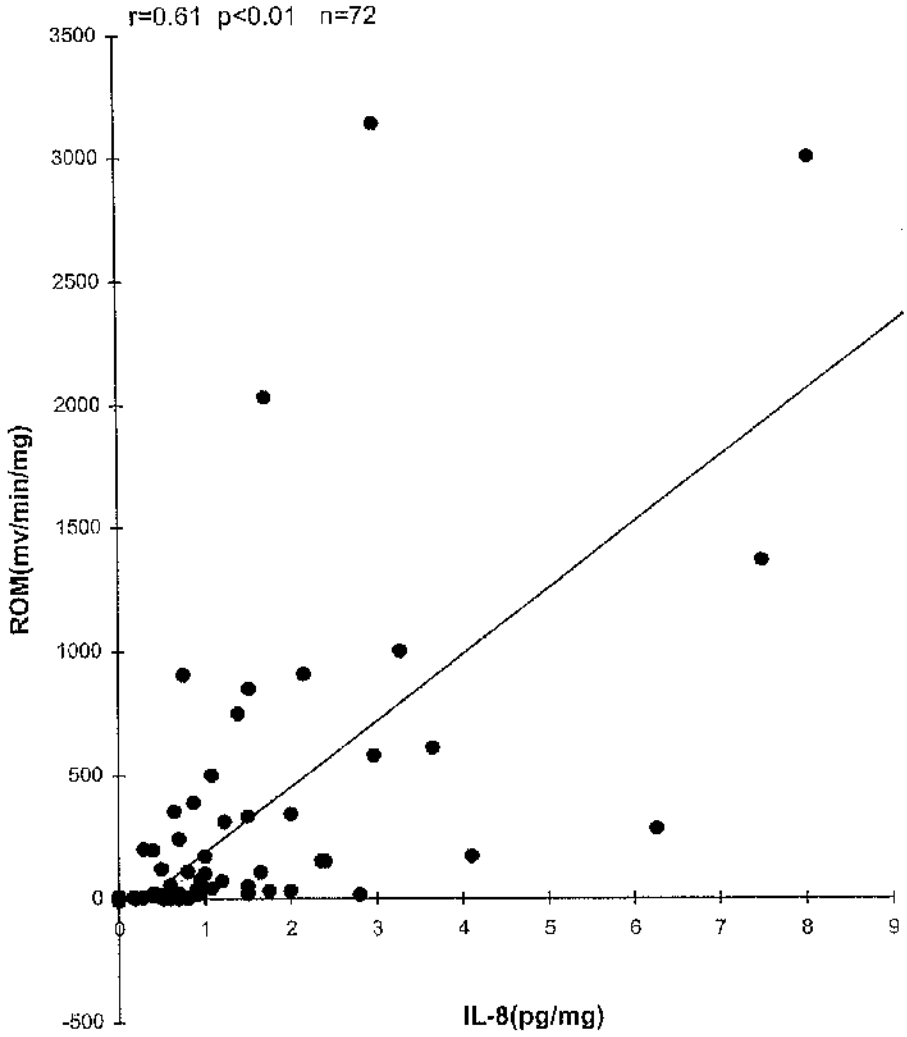


Fig 13 Association between gastric mucosal levels of IL-8 and ROM

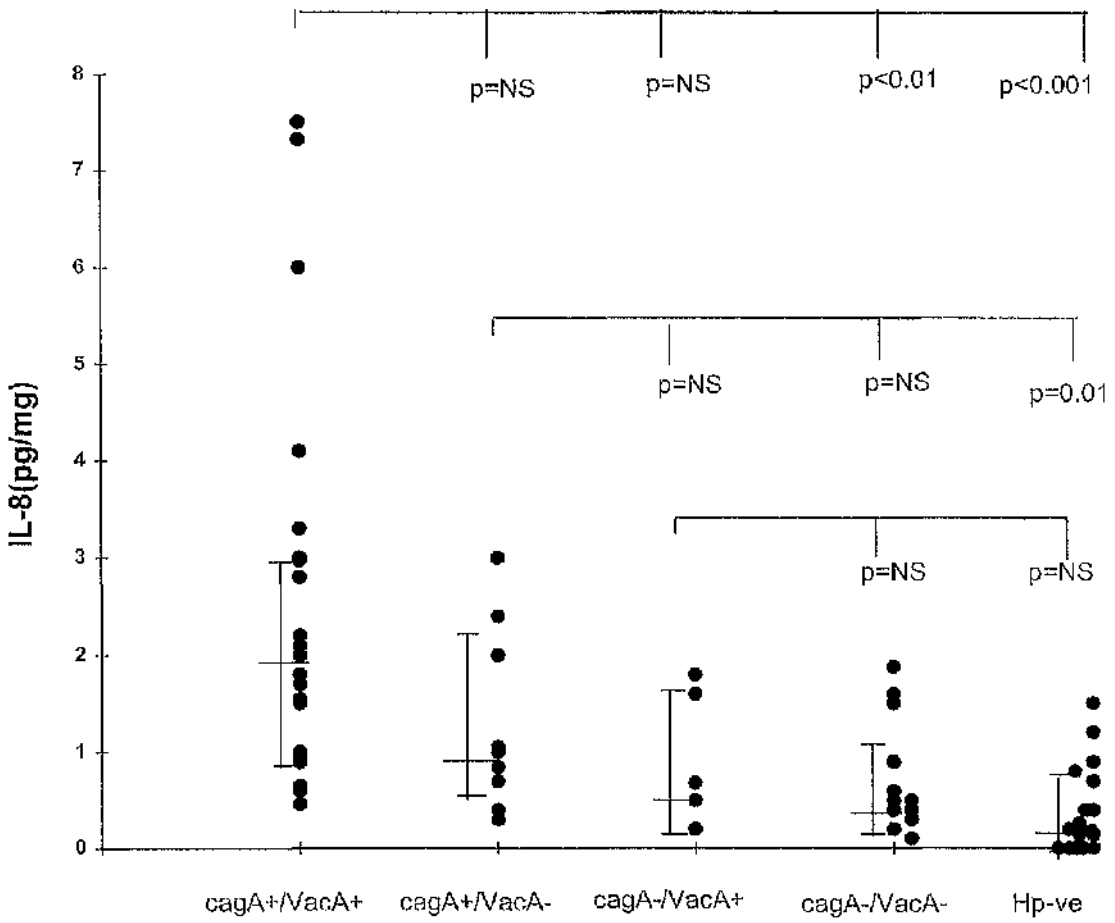


Fig 14 Relationship between mucosal levels of IL-8 and VacA expression and possession of cagA gene of H. pylori strains. Medians, interquartile range and p values are shown.

***Relationship between mucosal production of ROM and the expression of VacA and cagA of H. pylori***

Mucosal ROM levels(median(interquartile)mv/min/mg) were significantly higher in patients colonised with cagA+ve/VacA+ve strains(210(58, 730)) than that in those infected with cagA-ve/VacA-ve strains(30(8, 175), (p=0.004) and than that in those without *H. pylori* infection(0(0, 3))(p<0.0001). No significant difference was found in mucosal production of ROM between those patients infected with cagA+ve/VacA-ve strains and those infected with cagA-ve/VacA+ strains and those with cagA-ve/VacA-ve strains, although all those infected groups showed higher mucosal production of ROM than that in those patients without *H. pylori* infection(Fig 15, Table 10).

Table 10 Relationship between the presence of cagA gene and expression of VacA cytotoxin by strains of *H. pylori* and mucosal production of IL-8 and ROM.

| Patients' groups | Characteristics of strains | No | Median(interquartile) IL-8 (*pg/mg*) | ROM(*mv/min/mg*) |
|---|---|---|---|---|
| 1 | VacA+ cagA+ | 25 | 1.80(0.97, 2.99)* | 210(58, 730)# |
| 2 | VacA - cagA+ | 10 | 0.90(0.63, 2.10) | 104(21, 416) |
| 3 | VacA + cagA- | 5 | 0.60(0.28, 1.75) | 50(13, 1363) |
| 4 | VacA - cagA- | 12 | 0.50(0.35, 1.20) | 30(8, 175) |

* #p<0.05 compared to group 4 (cytotoxin-ve/cagA-).

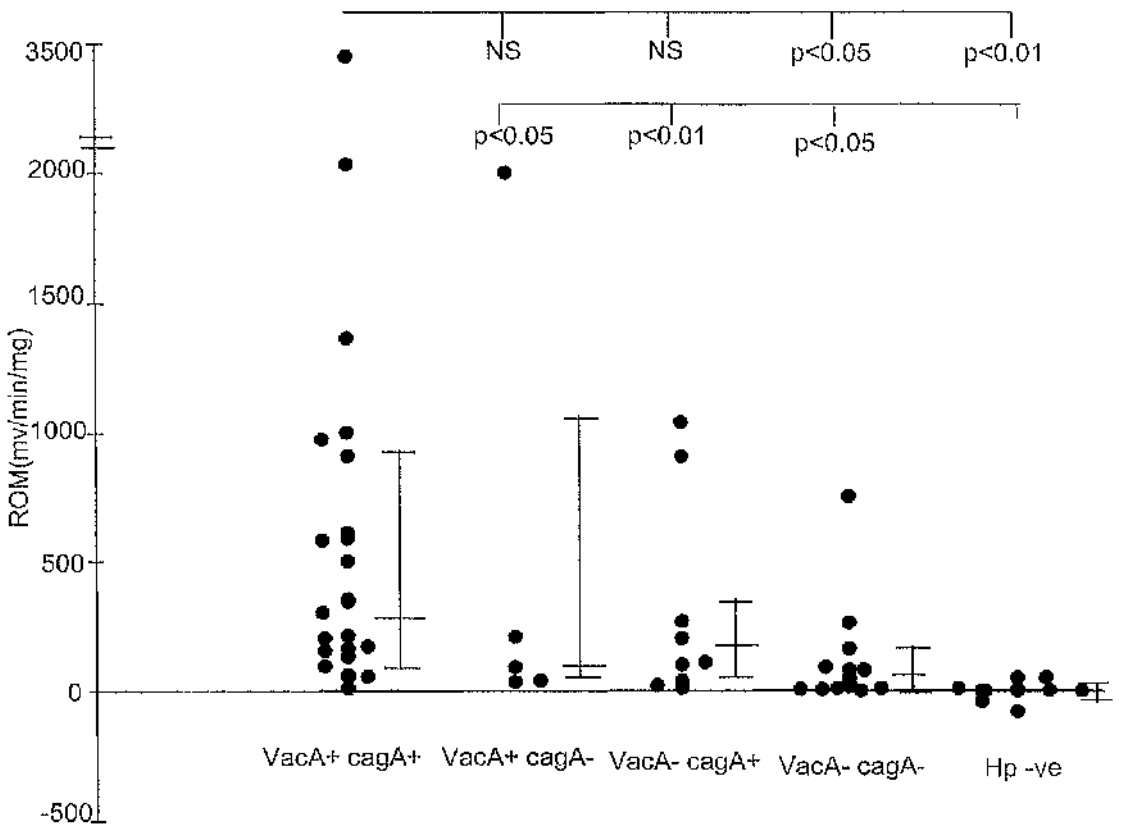


Fig 15 Relationship between mucosal production of ROM and expression of VacA and the possession of cagA of H. pylori. Medians, interquartile range and p values are shown.

***Relationships between IL-8 production and mucosal inflammation***

When antral mucosal inflammation was graded according to inflammatory cell infiltration, the mucosal IL-8 concentration(expressed as median pg/mg biopsy(interquartile)) was related to neutrophil infiltration: normal 0.20(0.0 to 0.77); mild 0.55(0.40 to 1.24); moderate 0.93(0.53 to 1.70); severe 2.25(1.06 to 4.30); (r=0.55, Fig 16). Higher IL-8 levels were also correlated with greater mononuclear cell infiltration: normal 0.10(0.00 to 0.35); mild 0.60(0.42 to 1.02); moderate 0.80(0.50 to 1.07); severe 1.50 (0.76 to 2.80);(r=0.42, $p<0.01$).

***Association of ROM release and histological inflammation***

Fig 17 shows that a greater CL response(expressed as median mv/min/mg biopsy(interquartile)) correlated with a higher infiltration of neutrophils: normal 0.0(0.0 to 12.3); mild 21.0(8.5 to 103.0); moderate 92.5(31 to 203.0); severe 556.0(152.0 to 1531.0);(r=0.51). CL response also correlated with mononuclear cell infiltration: normal 0.0(0.0 to 1.0); mild 9.0(0.0 to 26.5); moderate 16.2(5.0 to 43.5); severe 206.0(120.0 to 906.0);(r=0.39, $p<0.01$).

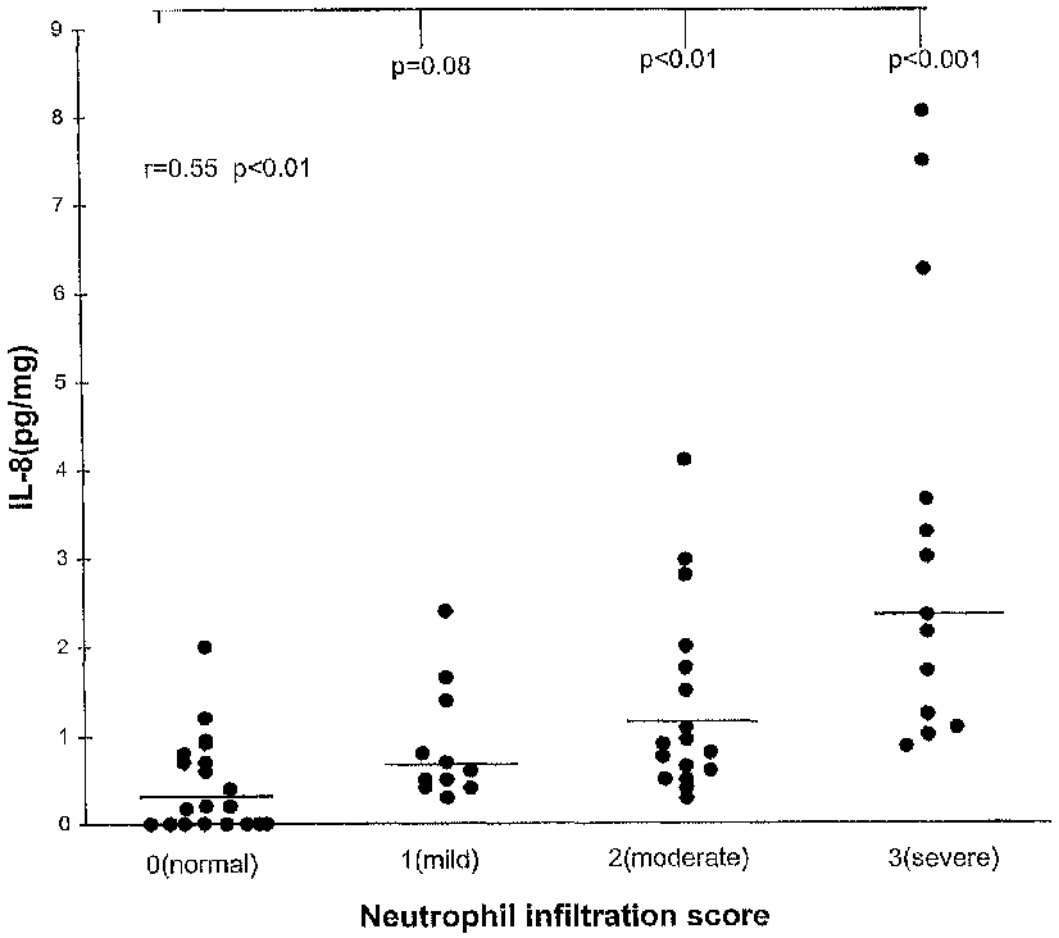


Fig 16 Association between antral mucosal production of IL-8 and histological score of neutrophil infiltration. Medians and p values are shown.

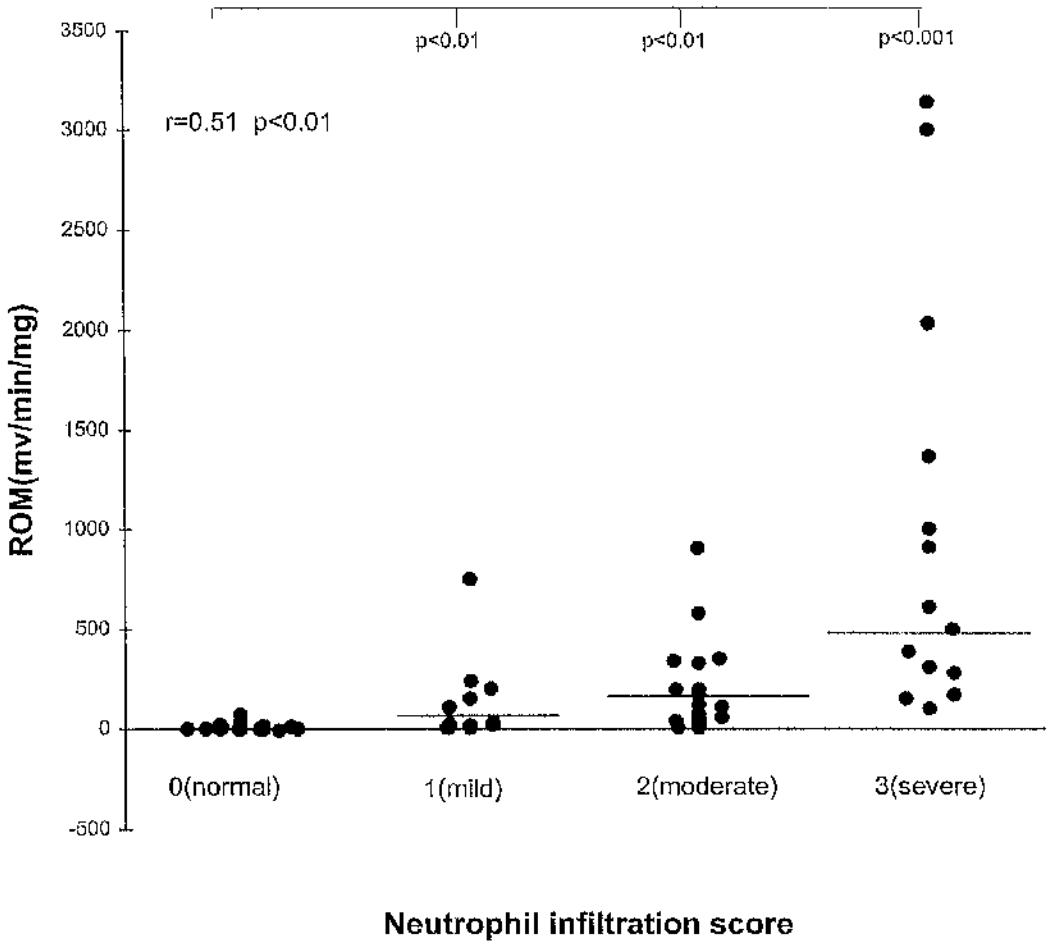


Fig 17 Antral mucosal production of ROM and histological score of neutrophil infiltration. Medians and p values are shown.

***Relationship between antral inflammation and expression of cagA gene and VacA***

When antral mucosal inflammation was assessed and scored according to the severity of infiltration of neutrophils and mononuclear cells, the neutrophil infiltration score was found significantly higher in patients infected with cagA+ve/VacA+ve strains(2.0(2.0, 3.0) than that in those infected with cagA-ve/VacA-ve strains(1.0(1.0, 2.0))($p<0.05$). There was no significant difference in mononuclear cell infiltration score between the two groups of patients(3.0(2.0, 3.0) vs 2.5(1.5, 3.0), $p>0.05$). There was no significant differences in inflammation score of neutrophils and mononuclear cells between patients infected with cagA+ve/VacA-ve strains and those with cagA-ve/VacA+ strains and those with cagA-ve/VacA-ve strains(Table 11).

Table 11 Relationship between bacterial virulence factors cytotoxin(tox) and cagA gene and antral mucosal inflammation.

| Patient groups | Characteristics of strains | No | Median(interquartile) inflammation score | |
|---|---|---|---|---|
| | | | Neutrophils | Mononuclear cells |
| 1 | Tox+ cagA+ | 25 | 2.0(2.0, 3.0)* | 3.0(2.0, 3.0) |
| 2 | Tox- cagA+ | 10 | 2.0(1.0, 3.0) | 2.0(1.5, 3.0) |
| 3 | Tox+ cagA- | 5 | 1.5(0.5, 2.0) | 2.5(1.25, 3.0) |
| 4 | Tox- cagA- | 12 | 1.0(1.0, 2.0) | 2.0(1.5, 3.0) |

* $p<0.05$ compared to group 4 (cytotoxin-ve/cagA-).

## 5.5 Discussion and conclusions

In this study, it was shown that both mucosal production of IL-8 and ROM are increased in *H. pylori* infected patients. There is also a good correlation between IL-8 concentration and ROM release in antral gastric mucosa. This suggests that local mucosal production of IL-8 in response to *H. pylori* infection does occur *in vivo* and may play an important role in attracting and activating phagocytes(both neutrophils and mononuclear cells) to release reactive oxygen metabolites; *H. pylori* itself may also secrete chemotactic factors for neutrophils (235,237).

It was shown that inflammatory cell infiltration is associated with mucosal production of IL-8 and ROM. This finding may have two implications. Firstly, it supports the hypothesis that IL-8 is actively involved in *H. pylori* induced inflammatory response by attracting neutrophils and mononuclear cells to the site of infection. Secondly, greater inflammatory cell infiltration and activation could produce more ROM and more extensive tissue damage[238,239,288].

Studies have been undertaken to investigate the possible component(s) of *H. pylori* that induces IL-8 secretion in vitro(214,270,271). In vitro studies have shown that cytotoxin+ve/cagA+ve strains induced greater epithelial cell production of IL-8 than cytotoxin-ve/cgaA-ve strains(269), suggesting that the $CagA^{+ve}/Tox^{+ve}$ phenotype of *H. pylori* is associated with enhanced IL-8 production. Subsequent studies using genetically defined cagA-ve or cagA-ve/vacA-ve isogenic mutants have suggested that neither cytotoxin nor the cagA gene product is directly involved in the stimulation of cytokine release(269,271). However, more recent studies suggest that the cagA gene is a marker for the presence of a group of genes(the *cag* pahtogenicity island) in type I strains, which may directly regulate the cytokine production(272).

Limited studies are available which address the relationship between bacterial type and *in vivo* mucosal production of IL-8. Peek et al(266) demonstrated increased cytokine expression in gastric mucosa infected with cagA positive strains of *H. pylori*; although the patient population studied were predominantly male of an older age and included a subgroup of patients who were taking NSAID. In the current study, we have investigated the relationship between bacterial types including cagA genotype and cytotoxin-producing status and the mucosal production of IL-8 in a general patient population with dyspepsia in whom NSAID use was excluded. It has been shown that patients infected with VacA+ve/cagA+ve strains had significantly increased mucosal levels of IL-8 than those infected with VacA-ve/cagA-ve strains of *H. pylori*. It was also shown that the former group of patients had a significantly higher neutrophil infiltration score than that of the latter group. These results support the in vitro finding that cytotoxin+/cagA+ strains induced an enhanced IL-8 production in epithelial cells(269) and the finding that patients with cagA+ve strains had heightened mucosal cytokine expression(266). There was no significant increase in IL-8 production in patients infected with cagA+ve/cytotoxin-ve strains over those patients infected with cagA-ve/cytotoxin-ve strains, although these patients showed higher IL-8 levels than those patients without *H. pylori* infection. This seems to contradict the in vitro finding showing that CagA+ve/cytotoxin-ve strains stimulated a significantly higher IL-8 production than those cagA-ve/cytotoxin-ve strains(270). The reason for this discrepancy between in vitro and *in vivo* findings is unclear. It is possible that there are differences between in vitro epithelial cell lines and *in vivo* human mucosal cells in their response to the bacterial stimulation. Although the expression of VacA(cytotoxin) by *H. pylori* is not a marker for the induction of IL-8,

its presence in combination with the cagA+ve genotype may represent a subgroup of strains of *H. pylori* which stimulate a stronger cytokine release *in vivo* and induce more severe mucosal inflammation and tissue injury.

In this study, 71% of cagA+ve strains were shown to express cytotoxin(VacA), and 83% cytotoxic strains were cagA+ve. These results were in accordance with previous reports that in about 70% of *H. pylori* strains, the cytotoxin expression and possession of the cagA gene are correlated(215). The genetic basis for this correlation is unclear. One study has reported that cagA status was concordant with vacA genotype in most strains studied: All cagA+ve strains were s1 vacA genotype; and all s2 strains were cagA-ve, whilst some cagA-ve strains were s1 vacA genotype(201). Atherton et al(201) has also shown the correlation between the cytotoxin(VacA) phenotypic expression and the vacA genotypes: s1/m1 genotype strains were high cytotoxin producers; s2/m2 strains were not toxin producers.

It was shown in this study that 80.6% of strains of *H. pylori* isolated from peptic ulcer patients were cagA+ve versus only 37.5% of strains from patients with non-ulcer dyspepsia(p=0.005), supporting previous findings that cagA+ve strains of *H. pylori* are more associated with peptic ulceration than patients with chronic gastritis alone(210). We have also shown that 66.7% of strains from patients with peptic ulcer were cytotoxin positive(VacA+ve) versus 37.5% of strains from patients with non-ulcer dyspepsia(p=0.045), supporting previous reports that cytotoxinogenic strains of *H. pylori* are more frequently found in patients with peptic ulcer disease(189-191).

It was also shown that patients infected with VacA+ve/cagA+ve strains had a higher mucosal production of ROM compared to those infected with VacA-ve/cagA-ve strains of *H. pylori*, supporting previous in vitro studies which

suggested that toxinogenic strains of *H. pylori* may induce an enhanced neutrophil oxidative burst(294,295). The results also showed that antral *H. pylori* colonisation density is an important determinant in ROM release, confirming the results by Davies et al(297) that the infective load of *H. pylori* is associated with the ROM production in the antral mucosa. Mucosal ROM production was associated with histological inflammation. These results would confirm the association between *H. pylori* infection and ROM production and support the role of ROM in *H. pylori*-induced mucosal damage.

Recent studies have suggested that *H. pylori* cytotoxin, encoded by the VacA gene, and the cytotoxin-associated protein(CagA) are important virulent factors, Both are considered to be important in the development of peptic ulcer. Clinical and genetic analysis supports the hypothesis that there may be more virulent or 'ulcerogenic' strains which are more likely to cause ulceration(201,210). A recent study by Atherton et al(206) has demonstrated some heterogeneity in the VacA gene, by showing that *H. pylori* strains of VacA signal sequence type s1a are particularly associated with enhanced gastric inflammation and duodenal ulceration. While the exact mechanism by which toxinogenic strains cause more severe mucosal damage is still unclear, increased production of mucosal ROM induced by toxinogenic strains may be important in this respect. In this study, we have shown that biopsies obtained from patients with duodenal ulcer had relatively higher levels of antral ROM than those obtained from *H. pylori* infected non-ulcer dyspeptic patients. This may be due to the fact that there were more toxin-producing strains in duodenal ulcer patients and these patients are more likely to have higher infective load of *H. pylori*(94).

In *in vitro* studies, several *H. pylori* products have been shown to activate phagocytes to release ROM, including a 150KDa protein(*H. pylori* neutrophil activating protein, HP-NAP), urease, lipopolysaccharides(LPS) and a 25-30KDa protein(175,236,293,296). It is as yet unknown which factor(s) is responsible for the *in vivo* stimulation of phagocytes. The vacuolating cytotoxin or the CagA protein *per se* is unlikely to be the major factor to induce a significant neutrophil activation(294). However, the results in this study that patients infected with VacA+ve/cagA+ve strains had relatively higher levels of mucosal ROM compared to those infected with VacA-ve/cagA-ve strains may suggest that the ability to induce phagocyte activation and mucosal ROM production is co-expressed or more strongly expressed in certain cytotoxic/cagA+ve strains of *H. pylori*.

In conclusion, gastric mucosal production of IL-8 is associated with an infiltration of neutrophils and mononuclear cells and is correlated with ROM release, which suggest that IL-8 may be important in attracting and activating phagocytes to release ROM thereby causing mucosal damage in *H. pylori* colonised patients. VacA+ve/cagA+ve strains are associated with an enhanced production of mucosal IL-8 and ROM *in vivo* and correlate with a stronger infiltration of neutrophils. The results support previous in vitro findings and suggest that enhanced mucosal production of IL-8 and its role in neutrophil chemotaxis and activation could be important in the development of gastroduodenal inflammation and peptic ulcer disease induced by VacA+ve/cagA+ve strains of *H. pylori*.

CHAPTER 6

# Mucosal levels of specific IgA, toxinogenicity of *Helicobacter pylori* and gastric inflammation

## 6.1 Introduction

Generation of mucosal IgA is an important local immune defence mechanism. *H. pylori* induces a significant IgA response in antral mucosa in infected patients(274,275). Despite the persistence of a local and systemic immune response, *H. pylori* is not eliminated. However, animal studies have shown that repetitive oral immunisation with *H. pylori* antigens and cholera toxin induced a strong local IgA anti-*H. pylori* response in mice and ferrets. Mice can be protected from infection by *H. felis* by immunisation with sonicated bacterial extracts or recombinant urease(299-301). Recent studies have also shown that oral administration of purified urease in combination with cholera toxin to mice or ferret, resulted in eradication of the bacteria in significant numbers of mice or ferrets(302,303). These results suggest that the mucosal IgA antibodies may have a protective role. Although the mucosal IgA antibodies failed to eliminate the organism in man, it may play a role in limiting *H. pylori* induced mucosal damage. Studies have suggested that cagA+ve or cytotoxin+ve strains of *H. pylori* are associated with a more serious pathology such as peptic ulcer disease and more active mucosal inflammation. There is little information available relating to specific IgA in response to toxinogenic and non-toxinogenic strains of *H. pylori* and disease activity.

## 6.2 Aims

To investigate whether any association exists between mucosal levels of *H. pylori* IgA, toxinogenicity of *H. pylori*, and mucosal inflammation.

## 6.3 Patients and experimental design

***Patients***

52 dyspeptic patients undergoing endoscopy were recruited. They include 28 patients with peptic ulcer(25 duodenal ulcer, 3 gastric ulcer) and 24 with non-ulcer dyspepsia(NUD). Patients who had had eradication therapy or taken non-steroidal anti-inflammatory drugs(NSAID) were excluded from the study. Patients were also advised to stop any anti-acid treatment two weeks before endoscopy. During endoscopy, biopsies were taken for histology, *H. pylori* culture and measurement of mucosal IgA *H. pylori* antibodies. Detailed methods were described in chapter 2.

## 6.4 RESULTS

***H. pylori infection and mucosal IgA H. pylori antibodies***

All 28 peptic ulcer patients and 10 of 24 of those with non-ulcer dyspepsia were infected with *H. pylori*. Antral mucosal IgA *H. pylori* antibodies were detectable in 35 of 38 patients with *H. pylori* infection, with a median(interquartile) level of 220.0(147.5, 531.0), while in those 14 patients without *H. pylori* infection, the IgA antibody was detectable at a low level in only one.

No correlation was seen between mucosal IgA *H. pylori* antibodies and patients' sex, smoking habits and alcohol consumption; nor was any found between patients' age and the antibody level, although in those below the age of 30 displayed a slight lower levels of IgA compared to those aged between 31 to 40 years(p=0.032)(Table 12). There was no significant difference in the mucosal IgA antibodies of patients with

peptic ulcer and those with non-ulcer dyspepsia infected with *H. pylori* (235(165, 620) vs 153(34, 345), NS).

Table 12 Demographic details of patients in relation to mucosal levels of *H. pylori* IgA(OD450nm×1000).

| Patient | No | *H. pylori* IgA (Median(IQ range)) | p value |
|---|---|---|---|
| Sex | | | |
| Male | 18 | 255(168, 643) | |
| Female | 20 | 190(45, 316) | 0.12(NS) |
| Age | | | |
| 20-30 | 7 | 69.0(0.0, 260.0) | |
| 31-40 | 12 | 313.0(154.5, 735.0) | *0.032 |
| 41-50 | 7 | 235.0(185.0, 659.0) | # 0.08(NS) |
| 51-60 | 6 | 219.0(128.5, 395.0) | # 0.12(NS) |
| ≥61 | 6 | 260.0(129.0, 539.0) | # 0.11(NS) |
| Smoking habit | | | |
| No | 16 | 240(160, 713) | |
| Moderate | 16 | 234(92, 420) | 0.8(NS) |
| Heavy | 6 | 230(130, 946) | |
| Alcohol consumption | | | |
| None | 20 | 197(115, 574) | |
| Moderate | 15 | 260(115, 574) | 0.6(NS) |
| Heavy | 3 | 144(128, 135) | |

* # compared to age group 20-30.

***Mucosal IgA antibodies and toxinoginicity of H. pylori***

*H. pylori* isolates from the 38 infected patients were analysed for the expression of VacA cytotoxin and the possession of cagA gene. Table 13 summaries the mucosal levels of IgA *H. pylori* antibodies in different patients colonised with *H. pylori* strains with or without the VacA or cagA. There was no significant difference in mucosal levels of the IgA antibodies between patients infected with cytotoxin+ve or cagA+ve strains and those with toxin-ve or cagA-ve strains of *H. pylori*.

Table 13 Antral mucosal levels of IgA *H. pylori* antibodies in patients with *H. pylori* infection with reference to toxinogenicity of strains

| Strains associated with infection | No | Mucosal IgA antibodies(OD*1000) (Median(IQ)) |
|---|---|---|
| Toxin +ve | 24 | 219.0(71.7, 348.0) |
| Toxin -ve | 14 | 260.0(148.0, 565.0) |
| | | p = 0.34(NS) |
| cagA +ve | 8 | 234.5(107.0, 510.5) |
| cagA -ve | 30 | 220.0(90.0, 505.0) |
| | | p = 0.51(NS) |

***Mucosal IgA H. pylori antibodies and mucosal inflammation***

When antral mucosal inflammation was assessed according to the degree of infiltration of neutrophils and mononuclear cells, there was no significant correlation between the mucosal levels of IgA antibodies and mononuclear cell infiltration

score(Fig 18). However, the IgA levels in those patients with severe neutrophil infiltration(neutrophil score: 3) were lower than those with mild and moderate infiltration(neutrophil score: 1 and 2)(Fig 19).

## 6.5 Discussion and Conclusions

It was found in this study that most infected patients had raised mucosal levels of *H. pylori* IgA. While there were large variations in the mucosal level of IgA among patients, there was no significant difference between patients infected with toxinogenic strains(either VacA or cagA) and those with strains not expressing either VacA or cagA. There was no significant correlation between patients' age and the mucosal levels of IgA; although a slight lower level of the IgA antibodies in patients below the age of 30 was apparent. Lack of correlation between the toxinogenicity of *H. pylori* and mucosal IgA suggest that the variation in host's immune response may be more important and the time for which patient had been infected may also be relevant.

Although animal studies have shown that oral immunisation with *H. pylori* antigens induced a significant secretory IgA response and protected mice from infection of *H. felis* and even eradicated the bacteria from the infected mice, the role of mucosal IgA antibodies in the immunopathology of *H. pylori*-related gastroduodenal disease in man is unclear. Mucosal secretory IgA has been suggested to be important in inhibiting antigen uptake, blocking bacterial adherence and motility of the organism and also in toxin neutralisation(278). It has been shown that the IgA inhibits the cell vacuolation induced by *H. pylori* cytotoxin in vitro(279). Previous studies also showed that *H. pylori* in gastric biopsy was coated with

IgA, but only a proportion of the bacteria were coated(273). So it is possible that some bacteria evaded the IgA defence mechanism for some reason. Current information on the association between the mucosal antibodies and the disease activity is limited. Serum IgG titres were shown to be correlated with the severity of inflammation both in the antrum and in the body of the stomach(280). Serum IgG responses to the 54KD heat shock protein and to the vacuolating cytotoxin were correlated with acute mucosal inflammation(281). Interestingly, the serum IgA responses to whole-cell sonicate of *H. pylori* and to the cytotoxin were inversely related to chronic inflammatory scores(281). In this study, it was found that patients whose mucosa was severely infiltrated with neutrophils displayed lower levels of IgA than in those patients whose mucosa had mild to moderate neutrophil infiltration. Previous studies have shown that toxinogenic strains of *H. pylori* are associated with a stronger mucosal inflammatory response. These results may suggest that the pro-inflammatory property of toxinogenic strains may induce more severe inflammation with intense neutrophil infiltration, which could damage the integrity of the mucosa(297,298) and compromise the mucosal IgA defence. Another possibility may be that an insufficient IgA response in some patients allows more bacterial colonisation and more severe mucosal damage.

In conclusion, *H. pylori* infection induced a significant local mucosal IgA response in most infected patients. The level of the IgA antibodies does not appear to be correlated with the toxinogenicity of *H. pylori*. However, the pro-inflammatory property of toxinogenic strains may induce severe inflammation which may compromise the mucosal IgA defence and rendering the mucosa susceptible to further damage.

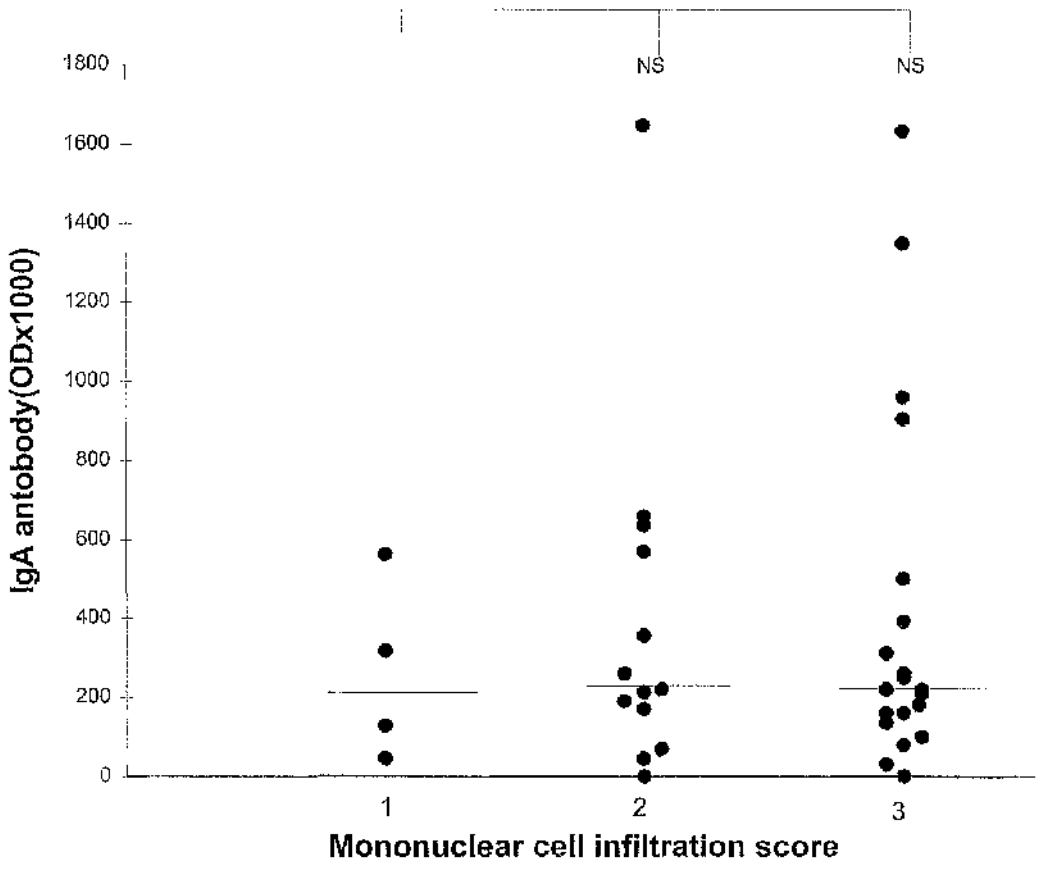


Fig 18 Relationship between mucosal IgA H. pylori antibodies and chronic mucosal inflammation. Medians, interquartiles and p values are shown.

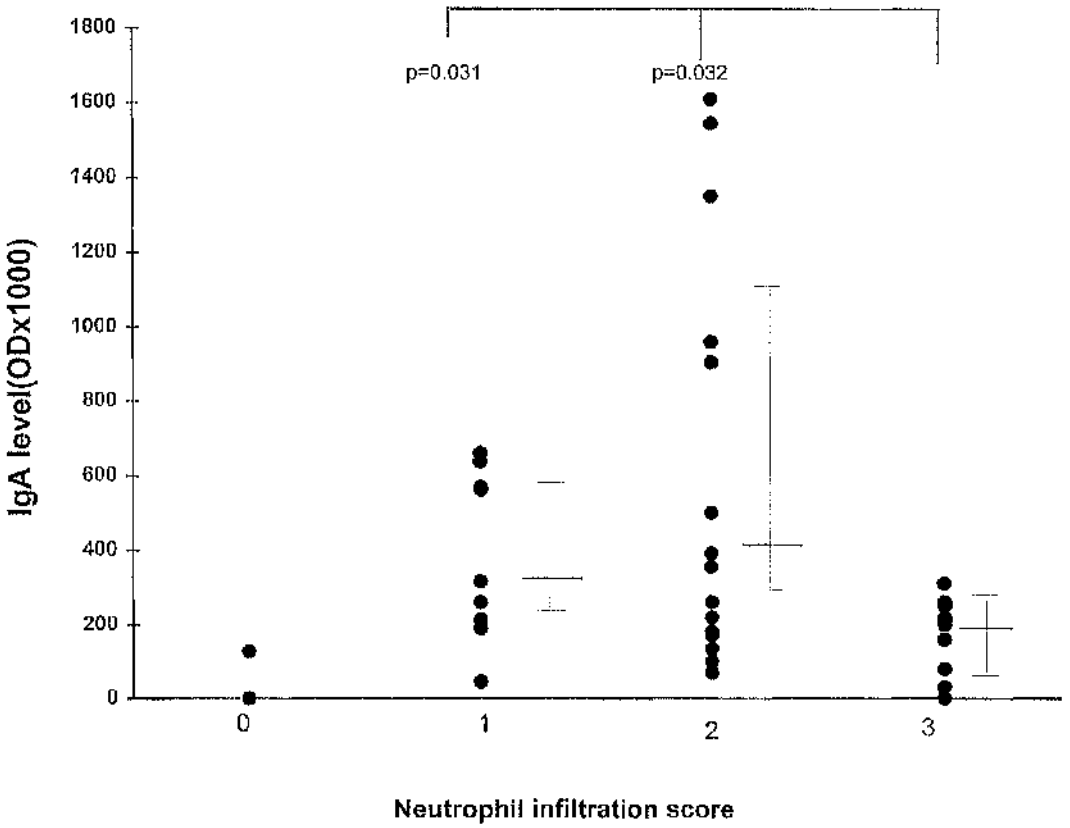


Fig 19 Relationship between mucosal IgA H. pylori antibodies and neutrophil infiltration score. Medians, interquartiles and p values are shown.

Chapter 7

# Effect of *Helicobacter pylori* cradication therapy on mucosal levels of interleukin 8, reactive oxygen metabolites and immunoglobulin A

## 7.1 Introduction

Recent studies have established the associations between *H. pylori* infection and chronic gastritis, peptic ulceration, and increased risk of gastric lymphoma of mucosa-associated lymphoid tissue(MALT) type and possibly gastric adenocarcinoma(1-4,98-103). Eradication of the bacterium leads to the significant reduction in the level of gastric inflammation, the cure of the ulcer and the regression in most low-grade gastric MALT lymphomas. Studies have suggested that *H. pylori*-induced local immuno-inflammatory reaction, including mucosal production of interleukin 8(IL-8), reactive oxygen metabolites (ROM) and immunoglobulin A(IgA), may be important in *H. pylori*-associated tissue damage(37,176,216,244,297,298). IgA is a predominant isotype of immunoglobulins in mucosal defence(304). *H. pylori* induces a strong local mucosal IgA response, although its role in the pathogenesis is unclear(274,277). There is little information on the effect of eradication of *H. pylori* on the mucosal immuno-inflammatory responses.

## 7.2 Aim of study

To investigate if changes occur after the eradication of *H. pylori* on the mucosal levels of IL-8, ROM and IgA.

## 7.3 Patients and experimental design

***Patients***

24 endoscoped patients with confirmed duodenal ulcer disease were recruited into the study. Patients who had received any previous *H. pylori* eradication therapy, those who had had previous gastric surgery and those receiving non-steroidal anti-inflammatory drugs were excluded. Patients had been advised to stop any anti-acid drug treatment at least two weeks before endoscopy. During endoscopy, gastric antral biopsies were taken using the same size forceps from similar topographical sites at each endoscopy for histology, *H. pylori* culture, CLO test, and chemiluminescence (CL, measuring ROM). For histological examination, inflammation and *H. pylori* colonisation were assessed by an independent pathologist.

***Measurements of IL-8 and IgA***

Each biopsy was weighed and placed in an Eppendorf tube containing phosphate buffered saline(PBS, pH 7.3). The volume of the PBS in each tube was adjusted to 1 mg of biopsy in 100μl of PBS. It was then homogenised separately by a tissue grinder at 4°C. The homogenate was centrifuged at 2000g for 10 min. The supernatant was removed and stored at -70°C, until the measurements of IL-8 and IgA.

IL-8 was measured by an ELISA(Bio-Whittaker, UK), following the methods described previously(Chapter 2) . The results were expressed as pg/mg biopsy. Mucosal *H. pylori* antibodies were measured by an ELISA(Sigma, St Louis, USA), in duplicate according to the instructions of the manufacturer as described in methods(chapter 2). The results were expressed as OD450nm×1000.

***Detection of reactive oxygen metabolites(ROM)***

Mucosal production of ROM was measured by Luminol-enhanced chemiluminescence(CL) as described in chapter 2. The results were expressed as mv/min/mg of biopsy after subtraction of the background.

***Treatment***

Patients with duodenal ulcer and infected with *H. pylori* were treated with lansoprazole 30 mg daily for four weeks, metronidazole 200 mg three times daily for one week and clarithromycin 250 mg three times daily for one week. Patients were invited to have a second endoscopy one month after completing the treatment and biopsies taken for the above tests.

***Statistical Analysis***:

Wilcoxon's matched pairs test was used to compare the differences before and after treatment.

## 7.4 Results

***Effect of treatment***

Before treatment, all 24 patients with duodenal ulcer had antral infection with *H. pylori*. After treatment, *H. pylori* was eradicated in 20 (83.3%); ulcer healing occurred in 22.

***Effect of eradication on mucosal levels of IL-8, ROM, IgA and mucosal inflammation***

In 20 patients in whom *H. pylori* was successfully eradicated, there was a significant reduction in median(interquartile) mucosal ROM(mv/mg/min) levels(153(53,595) to 20(0, 30), (<0.001) (Fig 20). There was a less prominent but significant decrease in mucosal levels of IL-8(pg/mg) (1.6(0.9, 3.0) to 0.6(0.1, 1.9), p=0.02)(Fig 21). There was a slight but not significant reduction in IgA levels (235(124,550) to 165(36, 265), p=0.06)(Fig 22).

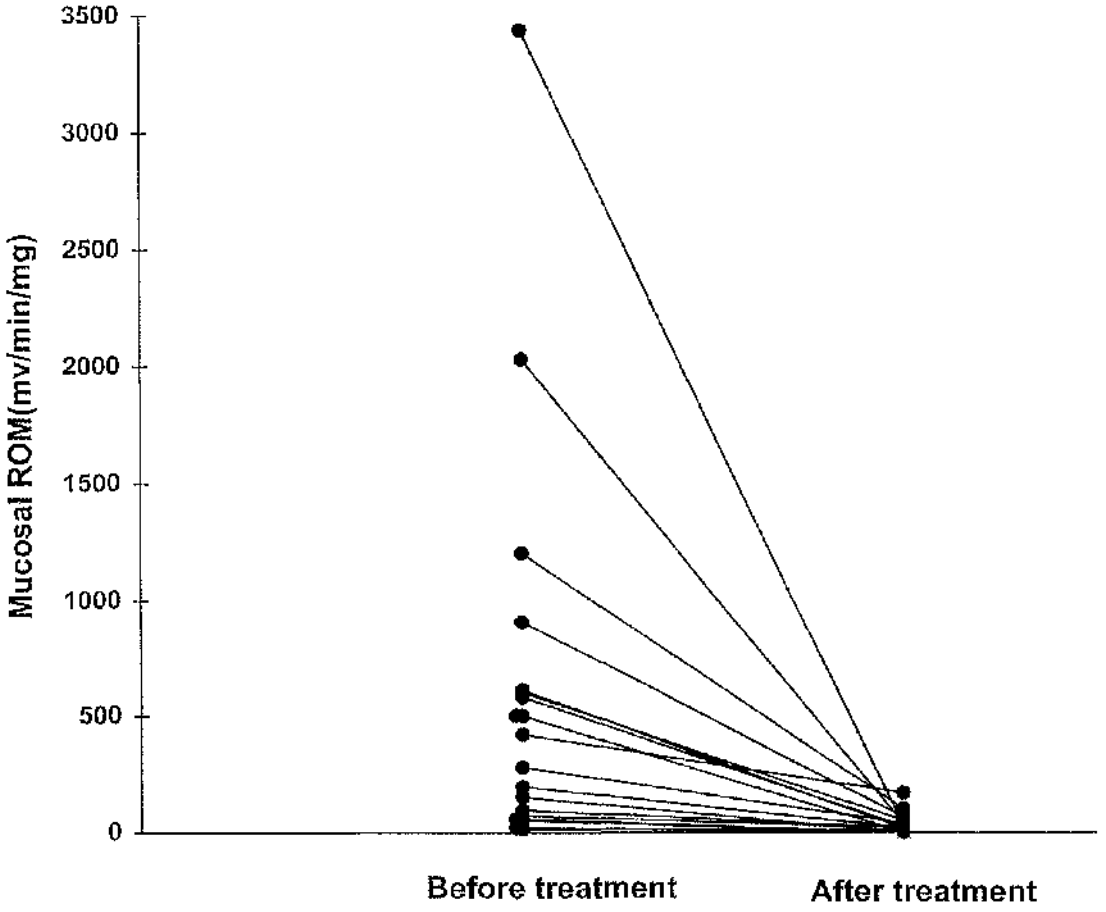


Fig 20 Mucosal production of reactive oxygen metabolites before and after eradication of H. pylori

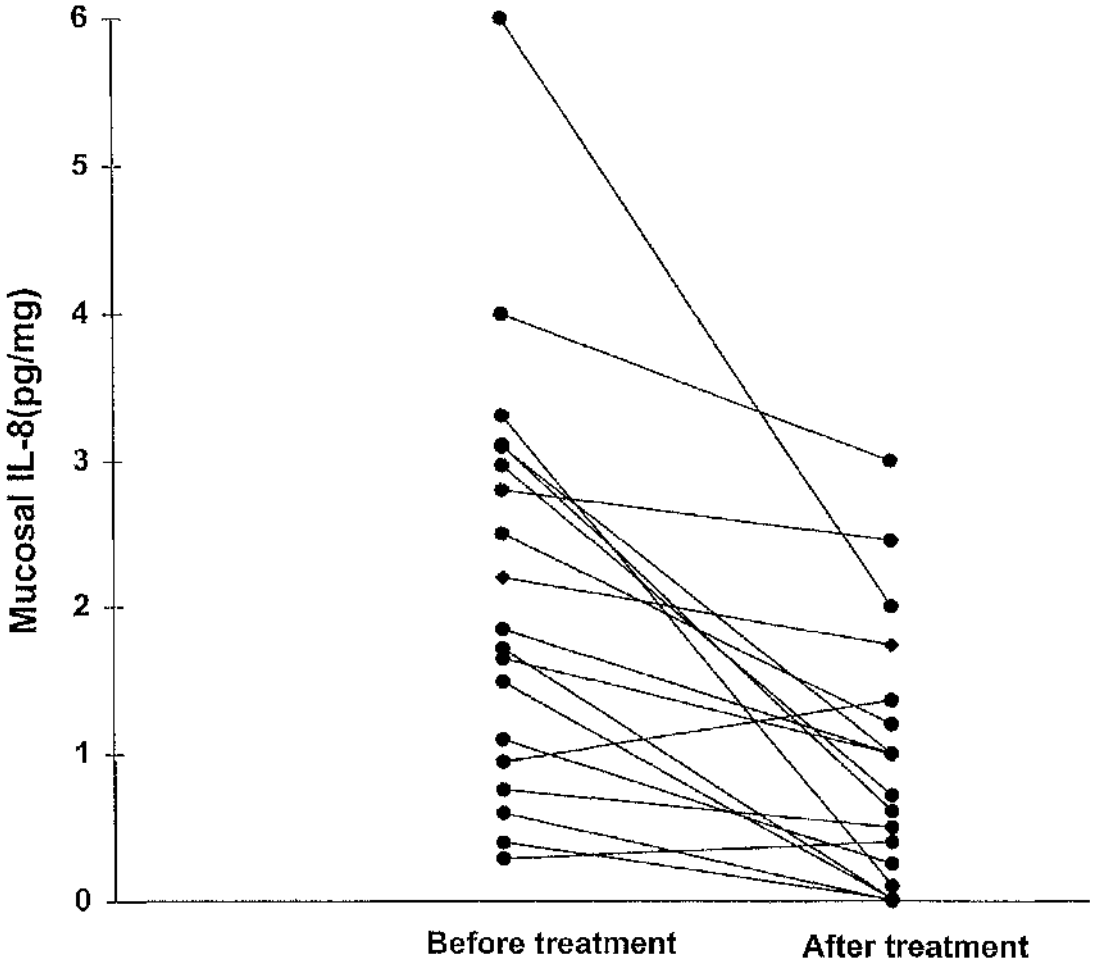


Fig 21 Mucosal levels of IL-8 before and after eradication of H. pylori

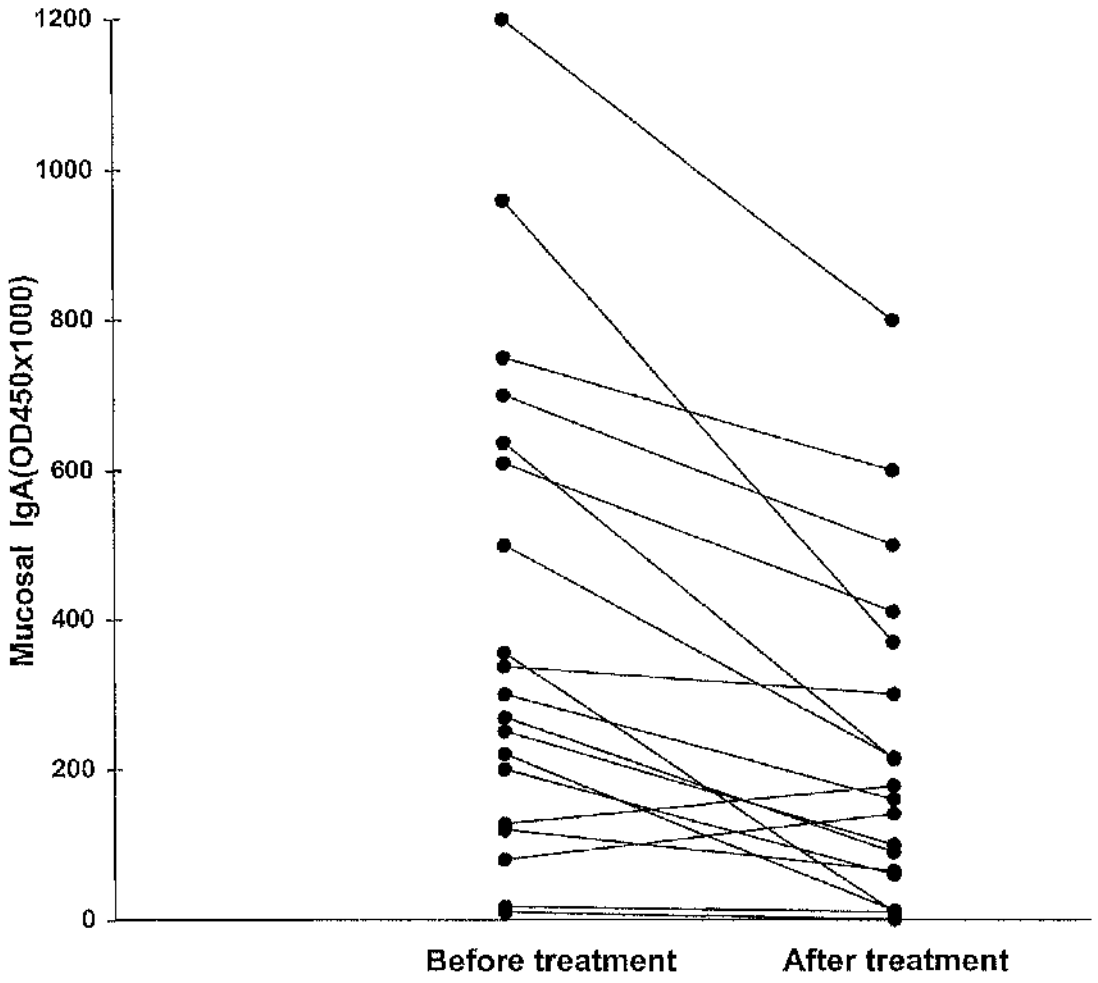


Fig 22 Mucosal levels of anti-H. pylori IgA before and after eradication of H. pylori

A significant improvement was seen in histological appearance with the virtual disappearance of the neutrophil infiltrate in all patients in whom *H. pylori* was eradicated ($p<0.0001$), and with a decrease in median(interquartile) mononuclear cell infiltration score from 2.5(2.0, 3.0) to 1.5(1.0, 2.0)($p<0.05$).

In those four patients who remained *H. pylori* positive after treatment, there were no significant changes in the levels of mucosal IL-8, ROM or in mucosal inflammation($p>0.05$). (Table 14)

Table 14 Mucosal levels of IL-8 , ROM and mucosal before and after treatment in four patients who remained *H. pylori* positive.

| | IL-8(*pg/mg*) | | ROM(*mv/min/mg*) | | PMN score | | Mononuclear cell score | |
|---|---|---|---|---|---|---|---|---|
| Patient | before | after | before | after | before | after | before | after |
| 1 | 1.6 | 2.0 | 200 | 500 | 2.0 | 2.0 | 3.0 | 3.0 |
| 2 | 1.0 | 0.9 | 180 | 150 | 2.0 | 1.0 | 2.0 | 2.0 |
| 3 | 2.6 | 4.0 | 340 | 360 | 3.0 | 3.0 | 3.0 | 3.0 |
| 4 | 1.8 | 2.0 | 974 | 480 | 3.0 | 3.0 | 3.0 | 3.0 |

## 7.5 Discussion and Conclusion

It was shown that one month after eradication of *H. pylori*, a significant reduction in mucosal ROM with a median from 153 to 20(mv/mg/min) was observed. There was also a moderate reduction in mucosal levels of IL-8 with a median from 1.67 to 0.61(pg/mg). Histologically, eradication of *H. pylori* is accompanied by the virtual disappearance of mucosal neutrophils and a moderate reduction in chronic

inflammatory cells. These findings support the roles of IL-8 and ROM in *H. pylori* infection. IgA antibodies to *H. pylori* were also reduced after eradication therapy with a median antibody titre from 235 to 165, although the IgA levels in some patients remained high.

A prominent histological feature associated with *H. pylori* infection is an infiltration of neutrophils and monocytes. Studies have suggested that *H. pylori* secretes certain products, which are chemotactic and/or capable of induction of the oxidative burst of phagocytes(235-237). *H. pylori* infection in the gastric mucosa also stimulates the mucosal cells to produce cytokines such as IL-8, IL-1, IL-6 and tumour necrosis factor(TNF)(261,264-267). These cytokines, especially IL-8 may be important in the trafficking and activation of phagocytes, which are a major source of ROM(238). The mucosal damage observed in patients with chronic gastritis may be caused by ROM(244,245). Therefore, if the above hypothesis is correct, eradication of *H. pylori* should substantially reduce the mucosal production of IL-8 and ROM. The significant decrease in ROM production seen in this study after successful eradication of *H. pylori*, accompanied by the disappearance of neutrophils and a moderate decrease in monocytes numbers, would confirm the importance of ROM in *H. pylori*-related mucosal damage, and would also support the notion the neutrophils are the main source of ROM. The mucosal levels of IL-8 were also reduced one month after completion of eradication therapy, but less significantly than ROM. The mucosal IL-8 concentration in some patients still remained relatively high one month after treatment. This finding contrasts with that of Moss et al(265) in which mucosal mRNA gene expression of IL-8 were determined and found to be quickly reduced to normal after eradication. The differences in the sensitivity of the methods used may

explain the apparently conflicting results(265). Previous studies suggested that IL-8 is extremely long-lived and is resistant to both high temperature and enzymatic degradation(252,257). In addition, a single stimulus may induce continuous production of IL-8 protein and persisting levels of IL-8 mRNA in vitro. It was also shown that the kinetics of IL-8 synthesis is prolonged in contrast with that of other cytokines such as TNF and IL-6(257,258). A biphasic production of IL-8 in lipopolysaccharides(LPS)-stimulated human whole blood was observed(257). A second phase of IL-8 synthesis, induced by early cytokine mediators(TNF and IL-1), lasted much longer than the first phase induced by LPS itself. So the slow decline in IL-8 levels seen in some patients after bacterial eradication may reflect the relatively prolonged kinetics of the cytokine.

Mucosal IgA is the most important Ig isotype in local immune defence. Although *H. pylori* induced IgA secretion does not appear to eliminate the infection, it may play some role in limiting damage caused by the bacterium. Animal studies using *H. pylori* urease preparations as oral vaccines against *H. pylori* infection suggested a protective role for mucosal secretory IgA(299-303). Previous studies have shown that the reinfection rate after eradication is very low in a two year follow-up(161,305). Another study by Miehlke et al(306) showed that the reinfection rate at 1, 2 and 5 years after eradication was 3.2, 3.2 and 9.7% respectively, and most of the duodenal ulcer relapses appeared to be associated with *H. pylori* reinfection. It was shown in this study that although the mucosal *H. pylori* IgA levels decreased after eradication, it remained high in most patients one month after eradication. Sustained synthesis of mucosal IgA after eradication may be important in preventing patients from reinfection.

CHAPTER 8

# General discussion and conclusions

The studies in this thesis have investigated the importance of *H. pylori* toxinogenicity and the host immuno-inflammatory responses in the pathogenesis of chronic gastritis and peptic ulcer.

Chapter 1 reviewed the current understanding about the pathogenesis of chronic gastritis and peptic ulceration, in particular the importance of *H. pylori* infection, and also the involvement of host immune response including neutrophil activation, mucosal production of IL-8, ROM, and immunoglob'ulin. Chapter 2 describes the general methods employed in the studies. The subsequent chapters have investigated: whether cytotoxin-producing strains of *H. pylori* are associated with the generation of an oxidative burst in neutrophils(chapter 3); the possible components of *H. pylori* which induce the neutrophil oxidative burst(chapter 4); whether there is any relationship between in vivo mucosal production IL-8 and neutrophil-derived reactive oxygen metabolites(ROM) release and whether mucosal levels of IL-8 and ROM are increased in those patients infected with toxinogenic strains(chapter 5); the relationship between mucosal IgA *H. pylori* antibodies and bacterial toxinogenicity and disease activity(chapter 6); and lastly the effect of eradication therapy of *H. pylori* on the mucosal levels of IL-8, ROM and IgA(chapter 7).

The important points can be summarised as follows:

1 Cytotoxin positive strains of *H. pylori* displayed an enhanced induction of the oxidative burst in neutrophils compared to toxin negative strains from patients with chronic gastritis only. Some non-cytotoxin-producing strains also induced a neutrophil

oxidative burst, suggesting that some component(s) other than cytotoxin itself may be responsible for the neutrophil activation. It is suggested that the neutrophil activating property may be more strongly expressed in most toxinogenic strains of *H. pylori*. The ability of some strains of *H. pylori* to produce cytotoxin and to induce the oxidative burst in neutrophils may be important in the pathogenesis of peptic ulcer disease.

2 Several *H. pylori* products mainly proteins may be involved in the activation of neutrophils. *H. pylori* cytotoxin and urease do not appear to be the active components for the neutrophil activation.

3 A good correlation between IL-8 concentration and ROM release in antral gastric mucosa suggests that local mucosal production of IL-8 in response to *H. pylori* infection may play an important role in attracting and activating phagocytes to release ROM. More intense inflammatory cell infiltration and activation may produce more ROM and cause more severe tissue damage. In line with the in vitro finding that VacA+/cagA+ strains induced an enhanced IL-8 production in epithelial cells, patients infected with VacA+ve/cagA+ve strains had significantly higher mucosal levels of IL-8 and ROM than those infected with VacA-ve/cagA-ve strains of *H. pylori*. The former group of patients also showed a significantly higher neutrophil infiltration score than that of the latter group. CagA+ve/VacA+ve strains may represent a subgroup of strains of *H. pylori* which stimulate a stronger cytokine release in vivo and induce more severe mucosal inflammation and tissue injury. More toxinogenic strains were isolated from patients with peptic ulcer compared to those with non-ulcer dyspepsia (NUD). The results support the hypothesis that there may be more virulent or 'ulcerogenic' strains. Enhanced mucosal production of IL-8 and ROM could be important in

VacA+ve/cagA+ve strains of *H. pylori*-induced gastroduodenal inflammation and in the development of peptic ulcer disease.

4 Although most infected patients had raised mucosal levels of IgA antibodies, there were big variations in the level of IgA antibodies among patients. There was no significant difference between patients infected with VacA+ve or cagA+ve strains and those infected with VacA-ve or cagA-ve strains. This suggests that differences in individual host response or some other bacterial factors other than cagA/VacA may be more important in stimulating an immune response. The time of infection may also be relevant. The finding that the mucosa with the highest infiltration score of neutrophils displayed lower level of IgA compared to the mucosa with mild to moderate infiltration may suggest that severe mucosal inflammation could damage the integrity of the mucosa and compromise the mucosal defence.

5 A significant reduction in mucosal ROM and IL-8 accompanied by a substantial improvement in histological inflammation support the roles of IL-8 and ROM in *H. pylori* infection. A sustained level of mucosal specific IgA after eradication of *H. pylori* might be important in preventing reinfection by the bacterium.

In conclusion, toxinogenic strains of *H. pylori* are more likely to induce a stronger host inflammatory response by inducing an enhanced neutrophil oxidative burst and an enhanced mucosal production of IL-8 and ROM, leading to a more prominent mucosal inflammatory cell reaction. More severe mucosal inflammation may damage the mucosal IgA defence, rendering the mucosa more susceptible to infection and injury. These findings may be important in the pathogenesis of *H. pylori*-related active gastritis and peptic ulcer disease.

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## PUBLICATIONS AND PRESENTATIONS

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