A STUDY ON IMMUNOLOGICAL AND BIOCHEMICAL CHANGES ASSOCIATED WITH *HELICOBACTER PYLORI* INFECTION

Thesis submitted to the University of Calicut
in partial fulfilment of the requirements
for the Degree of

DOCTOR OF PHILOSOPHY
IN MICROBIOLOGY

By

VIJAYAN K. T. V.

DEPARTMENT OF BIOTECHNOLOGY
UNIVERSITY OF CALICUT
KERALA, INDIA
April 2004

Dedicated to

my beloved parents

DECLARATION

I hereby declare that the work presented in this thesis entitled **A study on immunological and biochemical changes associated with *Helicobacter pylori* infection** submitted to the University of Calicut for the award of the **Degree of Doctor of Philosophy in Microbiology** is original and carried out by me under the supervision of Dr. M V Joseph, Head and Co-ordinator, Department of Biotechnology. University of Calicut. This has not been submitted earlier either in part or in full for any degree or diploma of any university.

CU Campus
26 April 2004

Vijayan K T V

ACKNOWLEDGEMENTS

I wish to express my indebtedness to my supervising teacher Dr. M V Joseph, Head and Co-ordinator, Department of Biotechnology, University of Calicut, for the proposal of the topic and the valuable guidance with incessant encouragement and meticulous efforts with critical suggestions to the successful completion of the work.

I accord my sincere thanks to Dr. T Rajeendranath, Baby Memorial Hospital, Kozhikode, Dr. Mohammed Zubair, Chest Hospital, Kozhikode, Dr. T Rajalakshmi of Aswini Diagnostics, Kozhikode, and also to the nursing and paramedical staff of Gastroenterology Unit of Baby Memorial Hospital and Operation theatre staff of Chest Hospital, Kozhikode.

I remember Dr. M H Nahm, Department of Pathology, Washington University, USA, with gratitude for providing monoclonal antibodies and subclass specific proteins. I convey my sincere thanks to Dr. V K Sasidharan and Dr. T Ramakrishna of Department of Life Sciences, University of Calicut, Dr. Ravindran Ankathil of Regional Cancer Centre, Thiruvananthupuram, and Dr. T Vijayakumar, Director, Centre for Health Sciences, University of Calicut for their timely help and encouragement. I extend my sincere thanks to Dr. Haridas, Sheffield University, UK, for the help rendered. I gratefully acknowledge Dr. P R Manishkumar and Mr. C. Gopinathan, Lecturers, Department of Biotechnology for the encouragement and help.

I accord my heartfelt thanks to Dr. K P Martin, Mr. P T Suneeshkumar and Ms. V Renuka of Department of Biotechnology, Dr. K P Rajesh of the Department of Botany, and Mr. Denoj Sebastian of the Department of Life Sciences for their benevolent helps to the successful completion of the thesis.

I convey my regards to the non-teaching staff of the Department of Biotechnology, Librarian in particular for being with the support all through the work. I extend my sincere thanks to the staff of the Department of Life Sciences, especially Mr. T C Rajasekharan, Technical Officer, and the Librarian.

I express my sincere gratitude to Dr. T Krishnakumar, Dr. E Sreekumaran, Ms. K Mini, Mr. C C Soundararajan (AIIMS, New Delhi), and Mr. Rahul Raghavan (St. John's Medical College, Bangalore), for their encouragement and timely help. I extend my thanks to the teaching and non-teaching staff, Mr. Rejadheesh in particular, of the Centre for Health Sciences, University of Calicut for the support and helps.

I gratefully acknowledge BRNS/DAE, Govt. of India for the financial assistance.

I extend my sincere thanks to Art and Photography Unit, University of Calicut for their assistance in the accomplishment of the photographs.

I accord my heartfelt gratitude to the enlivening support and inspiration of my beloved family members all through my life.

Vijayan K T V

CONTENTS

Chapter I

INTRODUCTION

CHAPTER I

INTRODUCTION

The frequent observation of bacteria-like organisms in biopsy samples of almost all patients with active, chronic gastritis, duodenal ulcer, or gastric ulcer inspired Marshall and Warren to obtain the culture of the organism *in vitro*. They succeeded in the process and isolated gram negative, microaerophilic, flagellated organism from human gastric biopsy samples (Marshall and Warren, 1984). The isolated organism was found to be a species related to *Campylobacters*. Initially it was given the name *Campylobacter pyloridis* and then renamed as *Campylobacter pylori*. But later studies revealed several properties unique to the organism and in October 1989 a new genus name *Helicobacter* was published (Goodwin et al., 1989).

HELICOBACTER PYLORI

Habitat and Morphology

In an attempt to prove Koch's postulates, two human volunteers swallowed the culture of *Helicobacter pylori* and demonstrated the ability of the organism to produce histological gastritis in a susceptible host (Marshall et al., 1985). The attempts to develop an animal model of *H. pylori* infections in germ free mice initially failed and this led to the speculation that the rodents may lack the attachment sites required for the colonization of human strains of *H. pylori* (Cantorna and Balish, 1990). The authors demonstrated the inhibited growth of *H. pylori* in presence of homogenates of murine stomach, small intestine and mesenteric lymph nodes. However, later several animal models were developed and Koch's postulates have been fulfilled for peptic ulceration and gastric cancer (Watanabe et al., 1998). Recently, guinea pigs were used as animal models and the *H. pylori* infection persisted in most of these animals for 5 months (Sjunnesson et al., 2003).

Helicobacter pylori was found in human gastric mucosa in association with gastric epithelial cells, at intercellular junctions (Hazell et al., 1986) and occasionally intracellularly (Noach et al., 1994). In untreated persons it is mostly observed in antral part and in persons treated with acid-suppressive drugs it may be present in higher numbers in the body of the stomach. Microscopy of the gram-stained smears or imprint of gastric biopsies demonstrated curved, gram-negative rods (Montgomery et al., 1988; Nichols et al., 1991).

On solid media *H. pylori* develop small, 0.5-1 mm diameter, transluscent, pale grayish colonies. In very young cultures the organism appear as almost straight rods microscopically. After 3-5 days of incubation, the bacteria look pleomorphic with irregular curved rods. In old cultures, they appear degenerative coccoid forms.

Goodwin and Armstrong (1990) reviewed the ultra structural features of the organism. Electron microscopy of thin sections and of negatively stained specimens showed *H. pylori* as unipolar, multiflagellate, rod-like organism with blunty rounded ends measuring 0.5 µm in width and 2.5-4.0 µm in length (Goodwin et al., 1985). The outer wall of *H. pylori* appeared smooth and closely following the underlying cytoplasmic membrane. This is a distinctive feature from true *Campylobacters*. A glycocalyx of 40 nm thickness, which is similar to the "S layer" in several gram-negative bacteria, is present on the surface of the organism. In gastric specimens, the surface glycocalyx of the organism and the epithelial microvilli are often linked by thread-like bridges or more extensive point of confluence. Four to six sheathed flagella are normally attached at one pole, each 2-5 µm long and about 30 nm thick. An extension of flagellar sheath as a membranous terminal bulb is observed. The flagellar sheaths are membranes and contain proteins and LPS (Suerbaum et al., 1993).

Virulence factors

In earlier days it was thought that stomach is free from infections due to its low acidity, thick layer of viscid mucus, gastric motility, and to certain extent the immune system of the body. But the successful isolation of *H. pylori* from gastric biopsies by Marshall and Warren (1984) abolished this concept. Due to the peculiar ecological niche prevailing in stomach, many scientists focused their studies on elucidating the factors contributing to the colonization of the organism in the stomach. In an article, Goodwin (1994) pointed out the following major virulence factors in *H. pylori.*

i) Urease: helps to overcome the gastric acidity

ii) Spiral shape and motility: allow organism to penetrate viscid mucus. In addition, various enzymes such as lipase, urease, and phospholipase may disorientate the micelles of the mucus structure.

iii) Adherence to mucosa and disruption of intercellular junctions helps to overcome gastric emptying.

iv) In mucus of the stomach immune cells such as polymorphs and IgG antibodies do not reach. Acid in stomach and pepsin destroy IgG. In addition, IgA blocking antibodies seem to adhere closely to the bacteria and this may impair opsonization by IgG and thereby impair phagocytosis. Thus, stomach becomes a sanctuary site for colonization of *H. pylori.*

Several authors reviewed the virulence factors of *H. pylori* (Wadstrom, 1995; Yoshiyama and Nakazawa, 2000). Broadly the putative virulence factors of *H. pylori* were divided into three groups: colonization factors, disease inducing factors, and factors promoting persistence.

Adhesion

Lingwood et al. (1989) observed recognition of a specific glycerolipid present in the extract of human RBC and pig stomach tissue by *H. pylori* isolates. This glycerolipid is found to be present in greater amounts in antral mucosa than fundal mucosa of human stomach and also more prevalent in gastric mucosa of adults than of infants. The specific recognition of lipid in gastric mucosa by *H. pylori* was suggested as the mechanism of colonization of stomach tissue. Kamisago et al. (1996) reported sulfatides and related sulfated compounds as major receptors for cell adhesion by *H. pylori* to the gastric mucosa. Saitoh et al. (1991) demonstrated the binding of intact *H. pylori* specifically to I^3SO_3 Gal cer and II^3 Neu-Ac-Lac cer extracted from human gastric mucosa and also the specific recognition of oligosaccharide moieties on these glycosphingolipids by different ligand molecules of *H. pylori.* Immunostaining revealed the significant localization of sulfatides in the epithelial lining of gastric mucosa (Saitoh et al., 1991). Huang et al. (1992) isolated two adhesin proteins of molecular weights 25 kD and 59 kD from *H. pylori.* According to these authors the receptor binding specificities and haemaglutination profiles of individual strains of *H. pylori* were attributable to the differences in antigenicity and molecular masses of these adhesins. It was reported that 5% of *H. pylori* acid-glycine extract proteins constituted the adhesive proteins (Ho and Jiang, 1995)

Wyle et al. (1990), by using transmission electron microscope, demonstrated strong association of *H. pylori* with the cultured KATO III cell lines and occasional cell evasion by the organism. Immunofluorescence and flow cytometric studies revealed that the adherence of the organism to KATO III cell lines were rapid, storable, energy dependent. and mannose resistant, but significantly inhibited by fetuin, a glycoprotein containing N-acetyl neuraminyl lactose (Dunn et al., 1991). Osaki et al. (1995), however, could not find any inhibition of *H. pylori* adherence to MKN 45 cells by fetuin. So involvements of different membrane receptors were suggested in the adherence of *H. pylori* to cultured

cells. Tight adherence of many *H. pylori* cells to individual KATO III cells resulting in direct juxtaposition of bacterial and cell membranes with indentation of KATO III cell membranes was demonstrated (Dunn et al., 1991). Logan et al. (1998) reported that 1-50 bacteria bound per gastric epithelial cells *in vitro*. They also observed adherence of all *H. pylori* strains to cultured gastric epithelial cells and the proportion of cells with bound bacteria varied from 40-99% while the proportion of cells bound with *H. cinaedi* and *H. fenneliae* varied from 5-15% only. In a study Wagner et al. (1994) used common drugs used for treatment of patients with gastric disorders and found that bismuth subcitrate blocked the adherence of *H. pylori* to epithelial cells while Omeprazole and Cimetidine do not interfere.

The effect of adherence of *H. pylori* on epithelial cells was demonstrated in Figure 1.1. Doig et al. (1992) purified a lamin binding protein having a molecular weight of 19.6 kD from *H. pylori*. The poor immunogenicity of this protein suggested its role in establishing persistent infection. The heterogeneity of *H. pylori* binding to tissue sections and inhibition of binding by synthetic Lewis^b oligosaccharides was reported (Reinhard et al., 2000). The findings of Suerbaum et al. (1993) suggested the role of proteins and LPS in the flagellar sheath in promoting the adherence of the organism to host cells.

From the view of colonization of *H. pylori* to the peculiar ecological niche in the stomach, it was suggested that the putative receptor responsible for *H. pylori* colonization should meet the following criteria: (i) present in mucus, (ii) specific for the mucus produced by gastric type epithelium, (iii) not present in the normal duodenum, (iv) not present in the mucus overlaying areas of complete intestinal metaplasia, (v) present in areas of gastric metaplasia in the duodenum. Some studies showed that MUC 5AC fulfills the necessary criteria in the stomach (van den Brink et al., 2000). The authors observed more than 99% of *H. pylori* association with either extracellular MUC 5AC or the apical domain of the MUC 5AC producing cells.

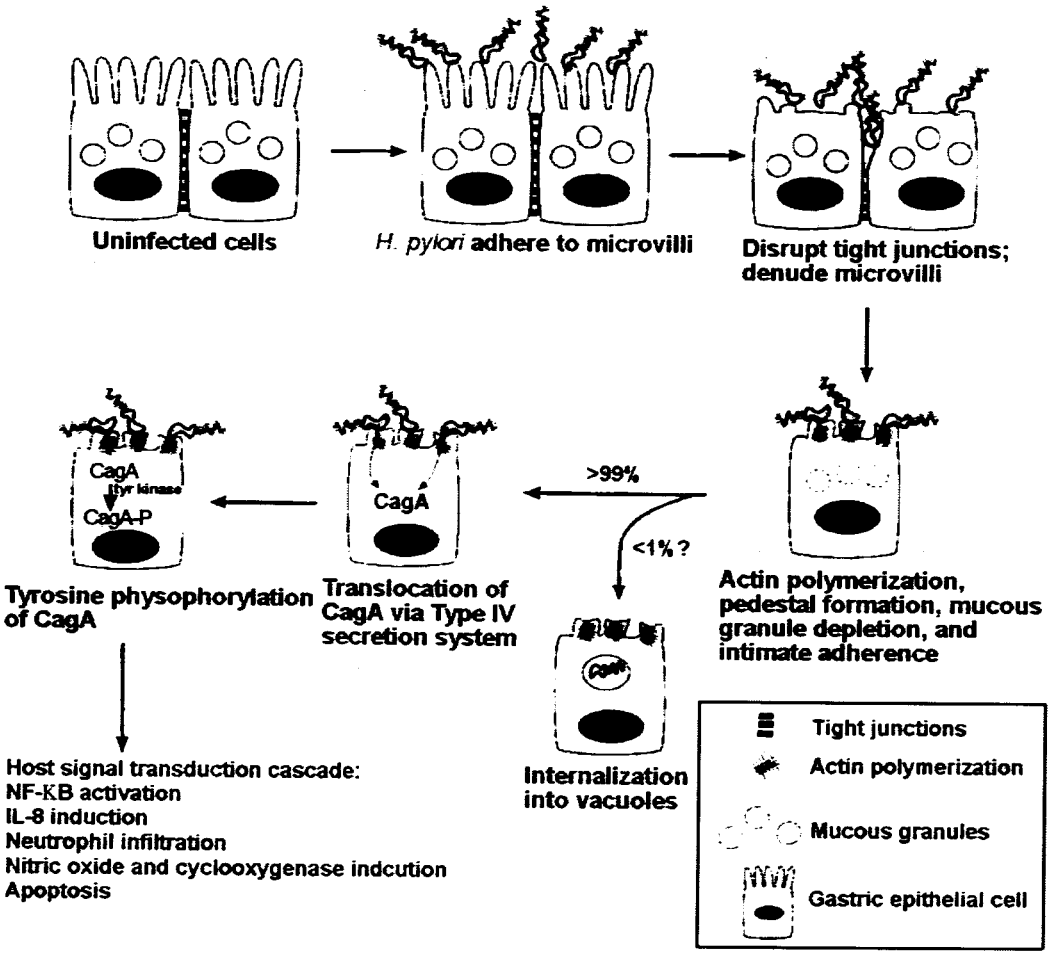

Figure 1.1 Effect of adhesion of *H. pylori* on epithelial cells

Biochemical activities

Urease

The gastric urease of human stomach was reported to be produced by *H. pylori* colonizing the gastric mucosa. Urease (Urea aminohydrolase: EC 3.5.1.5) is a high molecular weight, multi-subunit enzyme. Urease hydrolyses urea to ammonia and carbamate. Spontaneous decomposition of carbamate yields another molecule of ammonia and carbonic acid.

$$H_2N\text{-}CO\text{-}NH_2 + H_2O \xrightarrow{Urease} NH_3 + H_2N\text{-}C(O)\text{-}OH$$

$$H_2N\text{-}C(O)\text{-}OH + 2\ H_2O \longrightarrow NH_3 + H_2CO_3$$

In aqueous solutions, the released carbonic acid and the two molecules of ammonia are in equilibrium with their deprotonated and protonated forms, respectively. The net effect of these reactions is an increase in pH.

$$H_2CO_3 \longrightarrow H^+ + HCO_3^-$$

$$2NH_3 + H_2O \longrightarrow 2NH_4^+ + 2OH^-$$

Dunn et al. (1990) purified *H. pylori* urease and observed its similarities with urease of plants and other bacteria. Molecular weight estimated for native enzyme was 380±30 kD, where as subunit values of 62±2 kD and 30±1 kD determined. *H. pylori* produce urease in significant quantities under both *in vivo* and *in vitro* conditions.

Several reviews are available on *H. pylori* urease (Burne and Chen, 2000; Dick, 1990). Urease serves many functions in the metabolism of the organism, its colonization in gastric mucosa, and in pathogenesis of infection. According to Goodwin et al. (1986) and several others the key factor that protects the organism from gastric acid is urease. At low pH values intracellular urease generates enough ammonia from urea in the environment to maintain an ideal ecological niche for the organism (Marshall et al., 1990). They observed the protective effect of urea at a concentration as low as 0.05 mM/L.

Ferrero et al. (1988) observed that the activity of *H. pylori* urease was up to 20-70 times that of *Proteus vulgaris*, a urease producing bacteria. They also noted the glutamate dehydrogenase activity of *H. pylori* urease and suggested the assimilation of available urea nitrogen as a function of this enzyme. *H. pylori* urease does not cross-react with the urease of a closely related bacterium, St1, isolated from rodent ileum (Ferrero et al.,1988).

Most urease gene clusters contains ureABC genes, which encodes γ, β, α subunits respectively, that forms the urease apoenzyme. In *H. pylori* only ureAB genes are sufficient to encode urease (Labigne et al., 1991; Cussac et al., 1992). This was confirmed by the

studies of Hu et al. (1992) by transferring ureA and ureB genes encoding *H. pylori* urease into *E. coli* and subsequent transcription and translation of native size protein in *E. coli*. According to the authors additional modifications probably associated with Ni_2^+ insertion may be carried out by accessory genes downstream of ureB.

Biogenesis of functional urease requires the presence and expression of four urease accessory genes, ureDEFG. ureE acts as a nickel carrier, others are chaperones. In addition to the accessory genes, other genes are also involved in urease biogenesis. High affinity metal transporters, such as nixA gene of *H. pylori* scavenge the trace element nickel from the environment (Mobley et al., 1995). ureI gene encodes an integral cytoplasmic protein which is probably a pH activated urea transporter or perhaps regulates such a transporter as a function of periplasmic pH (Scott et al., 2000). Weeks et al. (2000) postulated that UreI form a urea specific pore in the cytoplamic membrane that opens at low pH and closes at high pH, thus regulating urea availability to cytoplasmic urease. Hong et al. (2000), using a contrast enhancement protocol in immunoelectron microscopy, demonstrated the localization of UreI antigen at or near the inner membrane of *H. pylori*.

Several authors reported the role of *H. pylori* urease in colonization of gastric mucosa and in pathogenicity of the organism. Eaton et al. (1991) demonstrated the inability of the urease mutant strains, which retained only 0.4% of urease activity of parent strain, to colonize piglets challenged orally, while the parent strain colonized all piglets and caused gastritis. Tsuda et al. (1994), by using nude mice, obtained similar results with urease mutant strains of *H. pylori*.

Perez-Perez et al. (1992) demonstrated the spontaneous development of urease mutants and that urease activity enhanced survival at acid pH. But according to Isogai et al. (1994) low pH and bacterial flora were not essential factors in establishing mucosal

infection with *H. pylori*. They demonstrated infection and consequent inflammation in the urinary bladder and pelvis of mice inoculated transurethrally with *H. pylori*.

Several authors demonstrated the role of urease in the pathogenicity of *H. pylori*. Smoot et al. (1990) reported the direct cytotoxicity of ammonia liberated by *H. pylori* urease on cultured gastric epithelial cells. The cytotoxicity was decreased when urease activity inhibited and ammonia concentration correlated directly with cell injury.

In addition to the direct deleterious effects of ammonia formation, other routes of cell damage have been proposed when urease or ureolytic bacteria interact with leukocytes. The formation of ammonia in the presence of oxidative burst created by immune cells can lead to formation of the monochloramines (Suzuki et al., 1992), which has been shown to be able to induce DNA damage. Moreover capacity of immune system to combat infection may also be compromised because of the inhibitory effects of elevated pH and ammoniacal compounds on immune function.

Spigelhalder et al. (1993) purified super oxide dismutase (SOD) from *H. pylori* and found that it has a role in preventing phagocytic killing of the organism. Catalase protects *H. pylori* against the damaging effects of hydrogen peroxide released from phagocytes (Hazell et al., 1991). Catalase negative mutants develop spontaneously *in vitro*, but have not yet observed *in vivo* (Westblom et al., 1992). This suggests that catalase is not necessary for the growth of *H. pylori* but it is important in the pathogenesis in the host.

Cytotoxicity

Leunk and colleagues (1988) discovered that supernatants from broth cultures of *H. pylori* induced massive degeneration of various cultured epithelial cell lines. The protein, which is mediating this effect was purified and named as vacuolating cytotoxin (Cover and Blaser, 1992). It was speculated that vacuolating cytotoxin (VacA) and ammonia generated by *H. pylori* urease may contribute to cell vacuolation and injury in additive fashion *in vivo*

(Cover et al., 1991). *In vivo*, ammonium chloride is generated from the combination of ammonia with HCl of parietal cell origin. Gastric juice ammonia in *H. pylori* infected persons was reported to be 34±16 mM (Marshall and Langton, 1986). The concentration of NH_4Cl that potentiated *H. pylori* induced cell vacuolation in cell culture assay was correlated with *in vivo* concentration of ammonia.

Studies on the structure and activity of VacA revealed that the mature secreted toxin have an approximate molecular weight of 90 kD and it contains two subunits or domains - a 37 kD amino terminal subunit and a 58 kD carboxy terminal subunit (Telford et al., 1994). The activity of the toxin was completely abrogated by removing 10 amino acids from the amino terminus and partially abrogated by removing 6 amino acids (de Bernard et al., 1998; Ye et al., 1999).

To find out the role of VacA *in vivo*, several animal studies were performed by using VacA toxin, wild type toxigenic strains, or vacA null mutants. The toxin caused damage to the gastric epithelium (Ghiara et al., 1995) but the colonization with wild type toxigenic strains and null mutants does not showed any difference in epithelial damage (Eaton et al., 1997; Wirth et al., 1998). Immunization of mice with VacA prevented infection when mice were subsequently challenged with *H. pylori* (Marchetti et al., 1995).

The precise role of VacA in peptic ulceration and gastric adenocarcinoma is still under investigation. It was thought that VacA may contribute to the capacity of *H. pylori* to colonize and persist human gastric mucosa and may also contribute directly to gastric epithelial damage. VacA can interfere with normal endocytic pathways, thus it may alter the process of antigen presentation, which may be one of the mechanism by which *H. pylori* evades host defenses (Molinari et al., 1998). While analyzing the genome sequence Tomb et al. (1997) proposed that VacA proteins may be retained on the outside

surface of the cell membrane and it might contribute to the interaction of *H. pylori* and host cells.

Crabtree et al. (1992) reported the association between expression of 120 kD protein, CagA, in *H. pylori* strains and cytotoxicity. Fallone et al. (2000) observed that the cagA gene was present in *H. pylori* isolated from 94-97% of subjects in all categories of patients with non-ulcer dyspepsia (NUD), gastroesophageal reflux disease (GERD), duodenal ulcer (DU), gastric cancer (GC) and controls. But the CagA antibody was less prevalent in NUD and GERD than those with gastroduodenal pathology including gastric ulcer (GU), DU, and GC. Results of some recent studies demonstrated that the infection with CagA positive *H. pylori* strains was associated with duodenal peptic ulcer in children (Queiroz et al., 2000). Infections with $VacA^{+}/CagA^{+}$ *H. pylori* strains were common in Chile and this phenotype was more prevalent in isolates from patients with more severe gastric pathologies (Figueroa et al., 2002).

Based on the expression of VacA and CagA all *H. pylori* strains could be partitioned into two groups, either which express (Type I) and do not express (Type II) cytotoxin and CagA proteins (Telford et al., 1994). Two basic Cag-mediated host cell signaling pathways that are induced upon attachment of *H. pylori* were differentiated, one is induced by the CagA protein, while the second pathway is independent of CagA (Stein et al., 2001).

Some recent studies demonstrated that CagE as a virulence factor associated with more severe disease outcomes after infection with *H. pylori* in children (Day et al., 2000). Isolates that possessed CagE induced greater chemokine response *in vitro* than did strains that were CagE negative. In strains of *H. pylori* isolated from patients with gastroduodenal pathology cagE gene and vacA s1 genotype were more frequent (Fallone et al., 2000).

Motility and Chemotaxis

Two crucial factors in the process of colonization of the host organism and establishment of successful infection by many pathogenic bacteria are motility and chemotaxis. The flagella of *H. pylori* have been extensively studied, and convincing evidences are available, which demonstrate the key role of these organelles in the colonization of gastric mucosa by the organism.

Eaton et al. (1992) observed the colonization of gnotobiotic piglets with motile strains of *H. pylori* up to 21 days while non-motile strains survived only for 6 days. The importance of motility of *H. pylori* was reviewed by O'Toole et al. (2000). It was thought that the flagellar sheath protects the acid-labile flagellar structure from the attack of stomach acid (Geis et al., 1993). Based on genome analysis, *H. pylori* possess a basic flagellum structure common to other bacteria with few variations. The variations reflect the niche adaptation peculiarities of small genome organism (Tomb et al., 1997).

In the *H. pylori* genome, about 10 genes have been identified that are assumed to be involved in the reception, transduction, and processing of chemotactic signals (Alm et al., 1999; Tomb et al., 1997). Foynes et al. (2000) demonstrated the importance of Che Y1 and Che Y2 and Che A in the motility and virulence of *H. pylori.* They found that Che Y1 and Che AY were necessary for flagellum regulated movement and chemotaxis towards mucin. Chemotaxis of *H. pylori* appeared to be distinct from the *Salmonella typhimurium* and *E. coli* paradigm. The absence of homologues of Che B or Che R in the genome of *H. pylori* supported this conclusion. The results obtained by Josenhans et al. (2000) strongly suggested a unique mechanism regulating motility in *H. pylori* which relies on slipped strand mispairing mediated mutagenesis of flip.

Lipopolysaccharide

Like the cell envelope of other gram-negative bacteria, that of *H. pylori* contains lipopolysaccharides (LPS). Although possessing properties similar to those of other gram-negative bacteria, the LPSs of *H. pylori* possess unique biological properties also. The attributes and structure of *H. pylori* LPSs, especially the relationship between molecular structure and pathogenesis were reviewed by Moran (2001). Slomiany et al. (1992) observed the detrimental effects of *H. pylori* LPS on mucus glycoprotein sulfation and assembly. They also showed the predominance of high molecular weight mucus glycoprotein in initial secretion of mucus glycoproteins on exposure to *H. pylori* LPS while the prolonged exposure led to the increase in low molecular weight mucus glycoprotein secretion. Increase in low molecular weight gastric mucin and decrease in mucin sulfation signals the onset of gastric disease.

Observations of Perez-Perez et al. (1995) indicated the low biological activity of *H. pylori* LPS in mediating macrophage activation. The ability of *H. pylori* LPS to induce cyclooxygensae 2 (COX-2) and the synthesis of prostaglandin E2 was showed to be lower than *E. coli* LPS (Smith et al., 2003). Thus it was speculated that the tight regulation of pro-inflammatory activities as a mechanism selected by *H. pylori* to maximize the duration of colonization.

Yokota et al. (2000) confirmed the presence of two distinct epitopes, highly antigenic and weekly antigenic, on the polysaccharide chains of LPS purified from clinical isolates of *H. pylori*. Based on the antigenicity of LPS in human beings, *H. pylori* strains can be classified into three types: (i) those with smooth LPS carrying the highly antigenic epitopes, (ii) those with smooth LPS carrying weakly antigenic epitopes, and (iii) those with rough LPS. Highly antigenic LPS were frequently observed in chronic gastritis patients.

while the isolates from the gastric cancer patients, especially tumour site isolates, more commonly showed low antigenicity (Yokota et al., 2000).

Considerable microdiversity exists in carbohydrate expression in the O side chain and LPS of *H. pylori.* This was demonstrated by using lectin typing and in *H. pylori* from Swedish population at least 16 lectin reaction patterns (MH 1 to MH 16) were identified (Hynes et al., 2002).

Young et al. (1992), using gastric mucosa of guinea pigs, observed a 50-fold stimulation of pepsinogen secretion with *H. pylori* LPS compared with only a 12-fold increase with *E. coli* LPS. Pepsinogen is a precursor of mucolytic and barrier breaking pepsin and its elevation is considered as a marker for the development and recurrence of duodenal ulcers.

Heat shock proteins

Several heat shock proteins (Hsp) produced by *H. pylori* such as 58.2 kD GroEl (Hsp B), 13 kD Gro ES (Hsp A) and 70 kD Hsp (Dunn et al., 1992; Evans Jr. et al., 1992). Yokota et al. (1994) reported the 66 kD proteins as the major heat shock protein of *H. pylori.* Hsp are produced by all cells and are involved in stabilizing and probably repairing proteins under harsh conditions that may be important to survive in the stomach. Yamaguchi et al. (1996) showed the epitope homology between *H. pylori* Hsp 60 and human gastric epithelial cells by using monoclonal antibody 3C8 and suggested that these homologous epitopes are important in the induction of gastroduodenal diseases by *H. pylori.*

Several authors reported the coccoid forms of *H. pylori.* Chan et al. (1994) reported coccoid forms in stomach. These coccoid forms are developing under certain conditions such as nutrient starvation and media containing growth inhibitors (Bismuth, proton pump inhibitors, or certain antibiotics). Eaton et al. (1995) demonstrated the virulence of coccoid

forms in gnotobiotic piglets. By using BALB/c mice Cellini et al. (1994) observed colonization and gastric ulceration with concentrated suspensions of coccoid forms. The data obtained by Osaki et al. (2002) suggested the role of coccoid forms in colonization and induction of mucosal inflammation.

A recent study identified a novel putative virulence factor, the oipA (outer inflammatory protein) gene, which encodes one of the outer membrane proteins (Yamaoka et al., 2000). It is an inflammation-related gene and the strains of *H. pylori* contain either a functional or non-functional oipA gene. The presence of functional oipA gene was associated with increased IL-8 production from a gastric cell-line and enhanced IL-8 production related to the cag pathogenicity island (cag PAI). Cag PAI, bab A2 or vacA status appear important only as surrogate markers for a functional oipA gene (Yamaoka et al., 2002)

From genome sequencing studies it was speculated that even though the organism possess a small circular genome, there are genes for many putative adhesins, lipoproteins, and other outer membrane proteins, which is consistent with the potential complexity of host-pathogen interactions. In addition to the microbiological etiology, other factors such as genetic predisposition, blood group, stress, drugs and smoking all have a role to play in the outcome of gastroduodenal pathologies.

Genetic diversity

The complete genome sequence of *H. pylori* is available now. It had shown that *H. pylori*, strain 26695, possess a circular genome of 1,667,876 bp and 1590 predicted coding sequences (Tomb et al., 1997). Average G+C content of the organism is 39%. Sequence analysis indicated a well-developed system for motility, for scavenging iron, and for DNA restriction and modification. It had a few regulatory networks and a limited

metabolic repertoire and biosynthetic capacity. In addition to chromosomal genome, *H. pylori* also possess small plasmid genome (Tjia et al., 1987; Penfold et al., 1998).

The genetic studies of vacA gene revealed enormous variations. The portion of vacA that exhibits maximum diversity is an ~ 800bp 'mid region' which encodes part of 58 kD domain of VacA and phylogenetically this region can be divided into two families of alleles, termed m1 and m2 (Atherton et al., 1995; Atherton et al., 1999). Aside from the vacA mid region, various other smaller regions, especially signal region, encoding part of the amino terminal signal peptide and amino terminus of the processed toxin exhibits striking diversity between strains. Two allelic families of signal regions are recognized, termed s1 and s2 and the s1 type can be further subdivided into s1a, s1b and s1c (Atherton et al., 1995; van Doorn et al., 2000). Subtyping of vacA signal and mid regions have given useful information on the population genetics of *H. pylori*.

The Lewis^x (Le^x), Lewis^y (Le^y) and related blood group antigens are present in the human gastric mucosa (Sakamoto et al., 1989). It has been speculated that bacterial expression of Le^x and Le^y antigens identical to those in the gastric mucosa may camouflage *H. pylori* in its ecological niche, particularly in the early phases of infection (Moran, 1996). Wirth et al. (1996) reported that *H. pylori* Le expression is related to the host's Lewis phenotypes. In *H. pylori*, Le^x is not a stable trait and LPS can display a high frequency of phase variation, resulting in the occurrence of several LPS variants in one bacterial cell population *in vitro* (Appelmelk et al., 1998). The protein profiling study of clinical isolates from *H. pylori* infected patients revealed that more than one antigenically different strains of *H. pylori* might exist in same infected individuals (Kitamoto et al., 1998). Phase variation could contribute to the phenotypic heterogeneity of *H. pylori* and may explain the isolation of several highly related isolates from one individual that differ in Le expression (Wirth et al., 1999).

Rasko et al. (2000) provided the first definitive evidence that a single strain of *H. pylori* may alter its LPS antigenic phenotype during the course of infection. In a follow-up study, Kuipers et al. (2000) observed a decrease in Le^y levels, but not Le^x levels in *H. pylori* strains. These observations demonstrated quasispecies in *H. pylori*, which may be considered the development, over time, of a pool of genetic variants from a single strain within a host providing a bacterial population that can be selected by the changing gastric environment of the host. The genetic drift occurring in the organism may be contributing not only to the evasion of immune mechanisms which enable successful colonization, but also to adapt to changing environmental conditions in the stomach and to colonize different compartments of the stomach.

Genetic diversity of *H. pylori* haemaglutinin/protease (hap) gene was reported. Hurtado et al. (1994) suggested that polymorphism in hap gene of geographically diverse strains of *H. pylori* may be mediated by transposon-like element. *H. pylori* hap gene is reported to be over 99% similar to *Vibrio cholerae* hap gene and the gene product is a secreted zinc metalloprotease having mucinase activity (Smith et al., 1994).

Epidemiology

Despite many interesting investigations and progress made, the epidemiology of *H. pylori* infections remain blanketed in fog. No reservoir for *H. pylori* has yet been found, except the human stomach. Sequence-based methods of strain comparison documented the co-evolution of *H. pylori* with human population (Bjorkholm and Salama, 2003). Taylor and Blaser (1991) reviewed the epidemiology of *H. pylori* infection. *H. pylori* are ubiquitously present and contact with the organism is rampant, but the incidences of infections are very low. This can be explained by the very high natural resistance to developing the infection in human beings by birth itself (Koster and Vanderbroucke, 1992).

H. pylori infection occurs worldwide, but there are significant differences in the prevalence of infection both within and between countries. In general, the overall prevalence of *H. pylori* infection in developed countries is lower than that in developing countries (Graham et al., 1991; Mitchell et al., 1992). This difference in prevalence is attributed to the rate of acquisition of *H. pylori* in childhood (Graham et al., 1991). The prevalence of *H. pylori* infection was very low in infancy, childhood and adolescence, and then quite suddenly rises somewhere between ages 20 and 40, after which the increase in prevalence per year slows down into some plateau (Perez-Perez et al., 1988). In France, few children were infected before age of 10, then the prevalence of infection increased gradually to 36.7% in the sixth decade of life. The same study showed increase in prevalence of infection with age in other countries like Algeria, Vietnam and Ivory Coast, but the rate was higher (80-90%) when compared to France (Megraud et al., 1989). A study in Southern China showed the diversity of living conditions as a prime determinant in the acquisition of infection (Mitchell et al., 1992). Transient *H. pylori* infection and re-infection after clearance is common among infants in high endemic areas (Casswall et al., 1998). The prevalence of *H. pylori* infection in children under the age of 10 years resident in developed countries were 0-5% compared with 13-60% in children resident in developing countries (Mitchell, 2001) and the significantly higher prevalence was among children in rural areas than in urban areas (Dore et al., 2002).

The increasing prevalence of *H. pylori* from younger to older subjects reflects the passage of the organism through the population of distinct cohorts. All persons are infected in childhood and the decreased levels of *H. pylori* infection associated with younger age groups, particularly in developed countries, are due to gradual improvement in medical care, sanitation and/or living conditions (Banatwala et al., 1993; Sipponen, 1995). It was reported that the acquisition rate of *H. pylori* was high and the subsequent chronic gastritis is common disease in cohorts born in the beginning of 20th century but both are much less

common in cohorts born more recently (Sipponen, 1995). In the early years of life spontaneous clearance of infection might occur (Malaty et al., 1999). The overall probability of acquiring *H. pylori* in a given 6 month period ranged between 0.28 and 0.38 and the probability of clearing the infection was between 0.22 and 0.45 in infants (Klein et al., 1994)

Increased seroprevalence of *H. pylori* infection in institutionalized persons gives evidence for person-to-person transmission (Harris et al. 1995; Proujansky et al., 1994). *H. pylori* status of the parents influences the *H. pylori* status of the offspring (Mertz et al., 2000). This may be due to the sharing behaviour and household exposure and vertical transmission. The presence of identical strains of *H. pylori* within family members supports familial transmission (Bamford et. al., 1993; Yamaoka et al., 2000). Close contact with infected individuals in early childhood results in acquisition of *H. pylori* infection, especially via contact with infected mother and other infected children (Miyaji et al., 2000). Infection by ingestion of *H. pylori* in vomitus or other objects was also suggested as possible mode of transmission by same authors. Children who are attending day care centers had higher prevalence of infection than did those who never attended. Children who had dogs were found to be at greatest risk of infection (Dore et al., 2002) and there are reports showing the isolation of organisms, which are homologous to human *H. pylori* from rhesus monkeys (Drazek et al., 1994).

Viable *H. pylori* were observed in gastric juice of infected persons (Young et al., 2000) and it was suggested that the gastroesophageal reflux introduces the organism into the mouth. 97% of patients tested by Song et al. (2000) showed the presence of *H. pylori* in the oral cavity with characteristic distribution that was independent of the infection status of the stomach. So it is thought that *H. pylori* may belong to the normal flora. Some workers obtained isolation of the organism from the faeces (Thomas et al., 1992) and dental plaques (Majumdhar et al., 1990; Oshowo et al., 1998). Gill et al. (1994) indicated

that *H. pylori* were present in the dental plaques of majority of children and their family members. Whether *H. pylori* is a resident or transient oral microorganism is still unclear.

The potential role of vectors in transmission of *H. pylori* infection was studied and Grubel et al. (1997) observed that the houseflies could harbour viable *H. pylori* on their body and their intestinal tract. But Osato et al. (1998) proved that *H. pylori* could not be recovered from houseflies fed human faeces either naturally infected or artificially infected with *H. pylori.* This study suggested that houseflies are neither a vector for transmission nor a reservoir for *H. pylori.*

Factors relating to the type of community in which the child lives may be as important as features of the family home (Patel et al., 1994). There are reports showing the association between the socioeconomic and educational level in the adults and water sources in the paediatric groups in acquisition of *H. pylori* infection (Olmos et al., 2000). Everhart et al. (2000) could not find any association of infection with water source, occupation, age at first sexual intercourse, number of sex partners, or cigarette smoking. Mendall et al. (1992) found that absence of a fixed hot water supply and domestic crowding in childhood as the independent risk factors for current infection with *H. pylori.*

The consumption of uncooked vegetables was found to be significant in *H. pylori* acquisition (Hopkins et al., 1993). Thus, it was suggested that the contamination of irrigation water with raw sewage and the subsequent contamination of the vegetables that are eaten uncooked is a key factor in the transmission of organism in Chile. Gill et al. (1994) suggested faeco-oral route of transmission of *H. pylori.* According to Mendall and Northfield (1995) faeco-oral transmission may be important in the developing countries and oro-oral transmission in the developed countries.

The prevalence of *H. pylori* infection shows ethnic differences. Everhart et al. (2000) observed 32.5% seroprevalence of *H. pylori* in the adult U.S population and age-

adjusted prevalence was substantially higher among non-Hispanic blacks (52.7%) and Mexican Americans (61.6%) than among non-Hispanic whites (26.2%). Male sex and education were associated with increased risk for seropositivity for non-Hispanic blacks compared with that for non-Hispanic whites. Yamaoka et al. (2000) reported 71% prevalence in Blacks, 74% in Hispanics, 77% in Whites and 97% in Vietnamese. Blecker et al. (1995) reported significantly higher overall prevalence of *H. pylori* in non-Whites (62.3%) when compared with Belgian-born Whites (17.8%).

The vacA genotype was found to be different in different populations. 93% of isolates from Vietnamese were s1c subtype while in Whites s1a subtype observed in 47%. In Blacks 80% had genotype s1 of these 93% were subtype s1b. vacA genotype m1 was most common in Blacks (69%), Hispanics (65%), and Vietnamese (55%). Whites showed vacA genotype m2 predominance (Yamaoka et al., 2000). In a seroprevalence study. Parsonnet et al. (1997) reported 79.4% of Blacks, 63.8% of Hispanics and 50% of Whites were positive for anti-CagA antibodies. These findings suggest that either genetic predisposition, or racial or ethnic groups to infections with particular *H. pylori* phenotypes or transmission of *H. pylori* within relatively segregated population groups. The studies of Malaty et al. (2002) confirmed that even within the same community, different cohorts might have different risks of acquiring the infection.

Perez-Perez et al. (2002) reported faster declining of $cagA^+$ seropositivity when compared to $cagA^-$ seropositivity over the 21 year period from 1973-1994, especially among subjects less than 45 years of age. They speculated that this difference might be related to differential acquisition and loss rates in later birth cohorts.

Relationship with Gastric Disorders and Gastric Cancer

The diagrammatic sketch of the human stomach is showed in the Figure 1.2. The regions of the ulcer development are also demonstrated in the Figure.

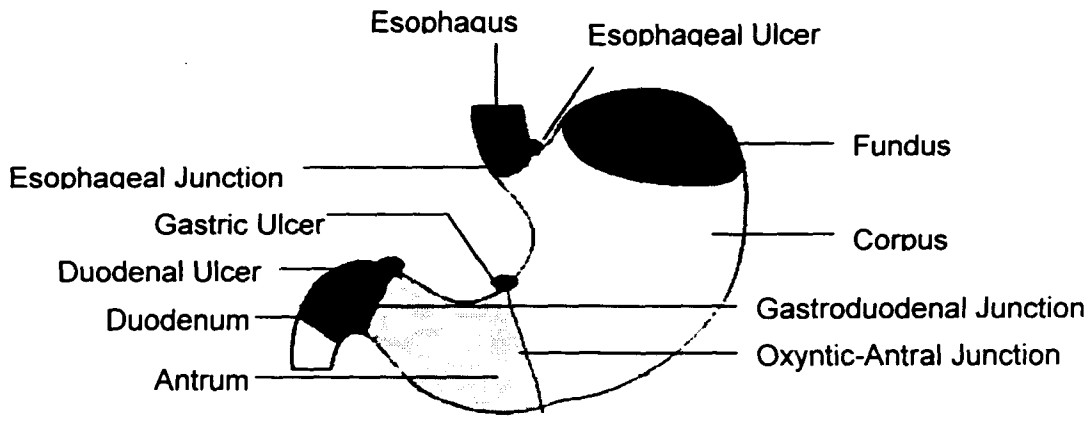

Figure 1.2 Gastric and duodenal anatomy

Chronic gastritis is a common denominator linking peptic ulceration, gastric carcinoma and lymphoma and the histological picture encompass chronic inflammation, atrophy and intestinal metaplasia. Our ignorance concerning etiology of these conditions led to a plethora of possible causes including stress, excessive alcohol intake, bile reflux and even the ingestion of hot beverages and spicy food. The findings of Marshall and Warren (1984) brought to light an explanation for whole series of these pathological changes. The accumulated knowledge of *H. pylori* pathogenicity and the host response to infection offers plausible explanation for the tissue changes observed and the ecology of the organism with regard to tissue colonization and local environment for differences in the patterns of inflammation seen in different clinical phenotypes.

The presence of *H. pylori* infection is an important marker for inflammatory gastroduodenal disorders (Blaser, 1990). Worldwide, approximately 50% of the population will be affected by the chronic gastritis during their lifetime of which more than 80% of chronic gastritis was associated with *H. pylori* infection (Sipponen, 1992). Megraud et al. (1989) compared the epidemiological data available on peptic ulcer disease in developing

countries with developed countries and speculated that the infection with *H. pylori* is not a sufficient condition to develop this disease. In India about 83% of population is exposed to *H. pylori* infection during the first decades of life (Gill et al., 1994). According to Prabhu et al. (1994), in Indians, histological gastritis is common and *H. pylori* infection is noticed in almost 25% of subjects with histologically normal gastric mucosa.

Sullivan et al. (1990) reported that infection with *H. pylori* is common in Gambia and in infancy, the infection is associated with chronic diarrhoea and malnutrition. Sullivan and Thomas (1991) reviewed the range of manifestations of *H. pylori* infection in children. In France, Raymond et al. (1994) observed that 75% of children with *H. pylori* infection and normal mucosa at endoscopy complained of recurrent abdominal pain.

The mammalian gastric mucosa, unlike other epithelial tissues has an intrinsic resistance to acid. The pioneering studies on gastric mucosal barrier was carried out by Davenport (1964, 1970) and the intrinsic resistance to acid is attributable to multiple properties of gastric tissue including its ability to: (i) secrete mucus and bicarbonate into the luminal space, (ii) dispose of, or neutralize back diffusing H^+ with specialized exchange pumps on both apical and basolateral membrane of surface epithelial cells, (iii) develop high electrical resistance across the plasmalemmal membrane at acid pH, (iv) rapidly reconstitute injured surface epithelium by the migration of immature foveolar mucus cells over the denuded surface, and (v) to maintain a hydrophobic luminal surface which in turn make the tissue non-wettable to corrosive actions of the gastric acid (Allen et al., 1993).

The organism has adapted to survive in the low pH environment of the stomach partly through its ability to buffer H^+ ions by the hydrolysis of urea and by the presence of lectins on its surface, which bind to gastric mucosa and epithelial cells (Collins, 1992). The factors that may alter the mucosal integrity include enzymatic and toxic reactions related to *H. pylori* infection, which may further trigger a number of immunological reactions in the

gastric mucosa and epithelium, as well as local production of free radicals as a result of mucosal inflammation (Sipponen, 1992).

H. pylori have evolved multifaceted acid-adaptive mechanisms enabling it to colonize the stomach. About 200 genes were up-regulated and approximately 100 genes were down-regulated at pH 4.5 in the absence of urea, and about half that number changed in the presence of urea (Wen et al., 2003). These genes included pH homoeostatic, transcriptional regulatory, motility, cell envelope, and pathogenicity genes.

Maaroos et al. (1994) were of the opinion that the *H. pylori* infection will decide whether the gastric ulcer disease will continue or heal spontaneously. Lichtenberger and Romero (1994) using rat as the animal model demonstrated the transformation of gastric mucosa to a wettable state by intragastric treatment with a combination of NH_4Cl and Muco-Mist. Such transformed mucosa was more sensitive to a challenge with physiological doses of HCl. Thus it was speculated that *Helicobacter* might induce attenuation in both mucosal hydrophobicity and barrier properties by producing high concentrations of NH_4^+ in the mucus gel layer. Murakami et al. (1995) observed that in *H. pylori* infected groups of patients, ammonia concentration in gastric juice and gastritis score in the antral mucosa were significantly higher than that in the *H. pylori* negative group. Following *H. pylori* eradication, the NH_3 concentration in the gastric juice and the gastritis score decreased significantly.

Both type I and type II *H. pylori* strains induced stress fibres in gastric epithelial cells and that were not observed in uninfected cells. Type I *H. pylori* induced cell elongation (humming bird phenotype) after 4 h of infection of epithelial cells *in vitro*, whereas the type II *H. pylori* strains did not (Su et al., 2003).

The acute phase of gastritis caused by *H. pylori* infection is subclinical in the great majority of subjects. Following ingestion, the organism penetrates through the viscid

mucus layer and multiplies in close proximity to the surface epithelial cells. *H. pylori* do not colonize all areas of the stomach. It will overlay only gastric type epithelial cells, whether normally present in the stomach or metaplastic in the duodenum (Buck et al., 1986). The epithelium responds to infection by mucin depletion, cellular exfoliation, and compensatory regenerative changes. Polymorph infiltration into foveolar and surface epithelium, and lamina propria edema are conspicuous. Collection of polymorph in the foveolae and adherent neutrophil exudates on the surface may also be present (Dixon, 2001). This acute phase is accompanied by profound hypochlorhydria and a failure of ascorbic acid secretion into gastric juice (Sobala et al., 1993). Rokkas et al. (1995) observed lower gastric juice vitamin C levels in gastritis patients and the decrease in level was correlated with the severity of gastritis.

The acute response to infection is mediated by a number of directly acting chemotactic moieties, which penetrate through the damaged surface epithelium and induce polymorph emigration into the lamina propria and epithelium (Crabtree, 1994). Nishiya et al. (1999) reported a higher grade of polymorphonuclear cell (PMN) infiltration in gastric mucosa infected with *H. pylori* strains, which are positive for iceA1 gene. Bacterial cells activate mast cells, and subsequent degranulation releases other acute inflammatory mediators that increase vascular permeability, up-regulate expression of leukocyte adhesion molecules on endothelial cells and increase polymorph emigration (Graham. 1992). A study in children revealed that cagA positive *H. pylori* were associated with a marked microscopic gastritis with a greater inflammatory infiltration of both mononuclear and polymorphonuclear cells in the antral and oxyntic gastric mucosa and degenerative and regenerative changes of the gastric mucosa (Queiroz et al., 2000).

The acute phase is short lived. In a small minority of people, especially in childhood, the organisms may be spontaneously cleared, the polymorph infiltrates resolves, and appearances return normal. In the majority, however, the host immune

response fails to eliminate the organism and over the next 3 or 4 weeks there is a gradual accumulation of chronic inflammatory cells that come to active chronic gastritis (Sobala et al., 1991).

Besides the bacterial virulence factors, the host response, with an increased mucosal production of inflammatory cytokines and reactive oxygen and nitrogen species could be relevant to the gastric pathophysiology in *H. pylori* induced duodenal ulcer (Klausz et al., 2003). *H. pylori* infection of the stomach induces a vigorous neutrophilic. T-cell and B-cell response. Humoral response will increase the antibody levels in the mucosa and gastric juice and systemic response increase the antigen-specific antibodies in the serum (Blanchard et al., 1999).

Similar to other infectious diseases, the first-line humoral response to *H. pylori* infection leads to the production of IgM antibodies. But this vigorous response fails to eliminate the infection. During the second line response in gastric mucosa as a result of persistent antigenic stimulation by *H. pylori* leads to an increase in IgA antibodies in mucosa and gastric juice. This is in accordance with the predominance (about 74-80%) of IgA producing B-cells in gastric body and gastric antrum (Sartor, 2002). The humoral response still fails to clear the infection and the formation of follicle becomes a consistent feature of chronic *H. pylori* gastritis.

Jarbrink et al. (2001) demonstrated the homing of lymphocytes in the stomach and found that it is similar to that occurring in intestine. Kutukculer et al. (1998) could not find any involvement of the local defense factors, sIgA and free secretory component, in the pathogenesis of *H. pylori* positive gastritis.

Rossi et al. (2000) observed that the experimental infection of Beagle dogs with *H. pylori* induces recruitment of gastric mucosae with neutrophils at early stages and later mononuclear cells that organize into lymphoid follicles. These structures became

macroscopically evident and consisted of peripheral $CD4^+$ T cells and central $CD21^-$B-lymhocytes. This acquisition of "organized" lymphoid tissue in the gastric mucosa constitutes mucosa associated lymphoid tissue (MALT) and it provides the background tissue in which MALT lymphoma arises. These findings underline the crucial role of *H. pylori* in lymphomagenesis in stomach (Dixon, 2001).

Stolte et al. (2002) reported the *H. pylori* and *H. heilmannii* gastritis as a pre-MALT lymphoma condition and noticed complete remission of the condition in 80% of low- grade lymphoma conditions by *Helicobacter* eradication therapy. Reappearance of acquired MALT after re-infection with *H. pylori* occurs in patients who previously had that condition (Tursi et al., 1997). The association between gastric nodularity and inflammatory response associated with *H. pylori* infection was reported by Elitsur et al. (2000).

Another sequelae of long-standing *H. pylori* infection are atrophy. Atrophy in stomach is conventionally defined as loss of glandular tissue from repeated or continuing mucosal injury and is common denominator in all pathological processes causing progressive mucosal damage. The loss of glands may follow erosions or ulcerations of the mucosa. Maaroos et al. (1994) observed that in both low and high-risk groups, corpus gastritis developed progressively into atrophic gastritis. Ohkusa et al. (2000) reported the greater histological severity of inflammation, neutrophil activity and atrophy in the antrum and corpus of *H. pylori* infected patients than in non-infected patients. The prevalence and severity of atrophy among patients with chronic *H. pylori* gastritis increases with time. Satoh et al. (1995) observed more extensive gastritis in groups of *H. pylori* positive patients aged 20-29 and 30-39 years than those without *H. pylori*. In their study the extension of atrophic gastritis began at 20 years of age in *H. pylori* positive while this began at 40 years of age in *H. pylori* negative subjects. They speculated that not only *H. pylori*, but also other factors, such as ageing, genetic and environmental factors are important in the chronological extension of atrophic gastritis.

Metaplasia, a potentially reversible change in which a fully differentiated cell type is replaced by another differentiated cell type, is frequently observed in chronic gastritis patients. Metaplasia usually represents a change to cells better able to withstand an adverse environment. Intestinal metaplasia represents a change from a gastric epithelial phenotype to a small- or large-intestinal phenotype. It was reported that *H. pylori* was found only in inflamed tissues and when the mucosa was atrophic or had intestinal metaplasia, dysplasia or cancer, the microorganism was not present (Guarner et al., (1993). Thus intestinal metaplasia can be viewed as a defense response against infection, but this interpretation remains speculative. Atrophic gastritis and intestinal metaplasia occurs predominantly at the gastric antrum and incisura with *H. pylori* infection. Antralization of gastric incisura is a common event in *H. pylori* infected patients and appears to be associated with intestinal metaplasia (Xiang et al., 2000). Guarner et al. (2003) described intestinal metaplasia associated with *H. pylori* infection in children and the authors also suggested atrophy in children since atrophy usually precedes intestinal metaplasia in adults.

Mucosal inflammation or the substances produced by *H. pylori* may impair gastric muscular function and cause a disorder in gastric emptying (Murakami et al., 1995). In dyspeptic patients without gastric phase III of the inter-digestive migration motor complex (MMC), the prevalence of *H. pylori* infection was higher and it was speculated that this abnormal motility might be a predisposing condition for bacterial colonization (Testoni and Bagnolo, 2000). Ohkusa et al. (2000) also reported the partial association of gastric emptying with *H. pylori* infection and they speculated the contribution of *H. pylori* infection to the development of NUD. The patients with perforated ulcer were infected with *H. pylori* more severely than those with haemorrhagic ulcer or stenotic ulcer (Tokunaga et al., 1998).

Patients with *H. pylori* infection had a significantly higher overall symptom score compared with *H. pylori* negative subjects. The severity of epigastric and nocturnal pain, heartburn, retro-sternal heartburn and vomiting was significantly higher in *H. pylori* positive functional dyspeptic patients and the influence on daily life and activities was significantly worse. Also loss of weight was significantly more common in *H. pylori* positive group (Werdmuller et al., 2000). Moayyedi et al. (2000) reported that *H. pylori* might be responsible for 5% of upper gastrointestinal symptoms in the community studies and *H. pylori* were significantly associated with dyspepsia.

A study in North India suggested that the patients with NUD have an increased incidence of *H. pylori* in gastric mucosa as compared with asymptomatic controls and they observed 54% of NUD patients were positive for *H. pylori*, while only 10% of controls had *H. pylori* infection (Mukohopadhyay, 1992). Antral predominant chronic gastritis and activity were reported in more than 90% of patients with *H. pylori* infection associated with DU, and in those patients the grade of gastritis correlated with density of organism (Kumar et al., 2002). Kumar et al. (1998) could not find any difference in the level of anti-CagA antibodies in DU and NUD patients in Indian population. So assessing anti-CagA antibodies cannot differentiate DU and NUD.

In a five years follow-up study Fujioka et al. (1995) observed that the rate of duodenal ulcer relapse was associated with the presence of *H. pylori*. Thus, the *H. pylori* eradication may be considered as a definitive cure for duodenal ulcer. The treatment of patients with a history of duodenal ulcer disease should be advocated, even if their ulcer disease is not "active" because the risk of recurrence and complications does not diminish unless *H. pylori* is eradicated (Neil and the Ad hoc committee on FDA related matters. 1997). In patients operated for duodenal ulcer, *H. pylori* infection was the only significant factor responsible for persistence of ulcer after surgery (Kumar and Sinha, 2002). The

authors recommended testing for *H. pylori* 6-8 weeks after surgery and institution of *H. pylori* eradication therapy in those found positive.

The pathogenesis of duodenal ulcer appears to be multifactorial, which involve an imbalance between "aggressive" (eg. acid and pepsin) and "defensive" (eg. prostaglandin production, blood flow, mucus/mucosal barrier, bicarbonate production, cell regeneration) factors. While reviewing pathogenesis of *H. pylori*, Peura (1997) concluded that there are a number of abnormalities in acid and gastrin physiology common to patients with DU, many of which may be related to the presence of *H. pylori*. Fasting serum gastrin levels were significantly higher in *H. pylori* positive patients than in negative subjects (Sumii et al., 1994). The basal serum gastrin levels were markedly decreased 5 weeks and one year after successful eradication of *H. pylori* but not in patients in whom treatment failed (Witteman et al., 1994).

H. pylori delivers powerful inflammatory signals to the host through activation of transcription factor Nf-κB. The sequential induction of chemokines, cytokines and expression of adhesion molecules in the target end organ facilitates the recruitment and proliferation of leukocytes to result in chronic gastritis and mucosal damage. Selection of Th1 dominant immune programme during infection undermines the protective ability of host response and further contributes to the disease pathogenesis (Ibrahimov and Pappo. 2000). Alkout et al. (2000) also observed enhanced inflammatory response and increased density of colonization of epithelial cells contributed to increased susceptibility of peptic ulceration. Lehmann and Stalder (1998) reviewed the role of cytokines in the peptic ulcer disease. According to them cytokines may be crucial in the recruitment and the activation of inflammatory cells and in stimulation of gastrin release.

Majority of the impoverished urban African population with gastroduodenal pathology were seropositive for *H. pylori* while none were seropositive for HIV even though

high HIV seroprevalence in the population. This study suggested that HIV infection was associated with protection against mucosal lesions and indicates that fully functional CD4 lymphocytes may be required for genesis of gastroduodenal pathology (Fernando et al., 2001).

Of the subjects tested by Mertz et al. (2000), 90% with elevated pepsinogen I (PG I) were seropositive for *H. pylori* compared to only 31% of those with normal PG I levels. PG I level correlate with maximal gastric acid out put and it was thought that increased PG I was a marker for increased acid secretion and DU in some families. Kikuchi et al. (2000) reported a decrease of PG I/PG II among *H. pylori* positive subjects and this was owing to a decrease in PG I in all subjects and an increase of PG II in those not younger than 30 years.

Tanahashi et al. (2000) indicated that the human gastric epithelial cells were involved in the mucosal inflammation that accompanied *H. pylori* infection and they have demonstrated the production of IL-6 and TNF-α, but not IL-8 by human gastric epithelial cells following stimulation with *H. pylori* urease. KATO III cell lines were found to be producing IL-8 and constitutively expressed ICAM-1 by the stimulation with live *H. pylori.* but not by *C. jejuni*, killed *H. pylori*. LPS. This led to the conclusion that *H. pylori* stimulated the gastric epithelium to initiate inflammation and neutrophil recruitment and activation (Crowe et al., 1995). The IL-8 induction by coccoid forms of *H. pylori* was significantly lower than that of helical form *in vitro* (Osaki et al., 2002).

H. pylori infection leads to up-regulation of the expression of surface Fas in gastric epithelial cells and the ligation of Fas of *H. pylori* infected cells with Fas L, which are produced by migrated neutrophils under the gastric epithelial cell layer. This can accelerate the apoptotic process (Kim et al., 2000). Dandekar et al. (2003) demonstrated transient.

Fas-mediated apoptosis in gastric lymphocytes, which is a compensatory response to the initial T-cell inflammatory response after acute *H. pylori* infection.

A considerable number of *H. pylori* infected patients have serum autoantibodies that are reactive with gastric mucosal antigens (Faller, 1997). Steininger et al. (1998) observed a positive correlation between apoptotic cell loss in the glandular zone of the corpus mucosa and the presence of anticanalicular autoantibodies (ACAB). In a 12 year follow-up study, Vorobjova et al. (2000) observed a significantly increased prevalence of ACAB in *H. pylori* positive patients. The authors also observed a significant age dependent increase in parietal cell antibody (PCA) in *H. pylori* positive patients. This established the association of ACAB with *H. pylori* gastritis particularly with atrophic gastritis. Claeys et al. (1998) identified gastric H^+K^+-ATPase as a major auto antigen in *H. pylori* associated anti-gastric autoimmunity. Earlier it was speculated that the site of inhibition of acid secretion by different strains of *H. pylori* possibly involves the H^+K^+-ATPase of the parietal cell (Jablonowski et al., 1994).

A decrease in gastric mucin synthesis *in vivo* may disrupt the protective surface mucin layer. Byrd et al. (2000) demonstrated the inhibition of total mucin synthesis and decrease in the expression of MUC 5AC and MUC 1 by *H. pylori in vitro*.

The combination of high duodenal acid load and *H. pylori* infection is likely the critical event in the pathogenesis of *H. pylori* related duodenal ulcer disease. Even though human bile inhibits *H. pylori* growth in dose dependent manner, the duodenal acid will favour *H. pylori* colonization by precipitating glycine-conjugated bile salts (Graham and Osato, 2000). In this context, they hypothesizes that any event that lead to an increase in the duodenal acid load will predispose to duodenal ulcer disease in patients with *H. pylori* infection.

It was observed that the smoking increases the duodenal acid load by increasing acid secretion and by inhibiting duodenal bicarbonate secretion. Thus, smoking may contribute to ulcer development in *H. pylori* positive persons (Graham and Osato, 2000). Chan et al. (1997) have also reported the facilitatory role of smoking in the pathogenesis of *H. pylori* related ulcers, but smoking did not increase the recurrence of peptic ulcers after eradication of *H. pylori*. Ogihara et al. (2000) reported a negative association of smoking with *H. pylori* infection. In their study the risk of *H. pylori* seropositivity was found decreased linearly with cigarette consumption per day. The authors also noticed the negative and dose-dependent association of drinking with *H. pylori* seropositivity.

The experiments of Alkout et al. (2000) revealed that the blood group O leukocytes bound significantly more bacteria of each strain and overall, released significantly higher levels of IL-6 and TNF-α than did leukocytes from other blood groups. The report underlined the role of enhanced inflammatory response to *H. pylori* pathogenesis of peptic ulcer disease. Both direct bacterial cytotoxicity and inflammatory cell aggression against gastric epithelium may predispose the patient to peptic ulcer disease (Fiocca et al., 1994).

The iron metabolism in humans is affected by *H. pylori* infection. Milman et al. (1998) observed reduced serum ferritin levels in people with increased IgG antibodies to *H. pylori*.

Molecular studies suggested the existence of disease specific cell lineages or strains which leads to the various outcomes observed in patients with *H. pylori* infection (Go and Graham, 1994). While reviewing the hypothesis that diversity among *H. pylori* strains and variability in the outcome of infection Blaser (1994) concludes the major possibilities into four categories: (i) virulence differs between strains of *H. pylori*. (ii) response to *H. pylori* differs between hosts, (iii) specific environmental cofactors modify

the *H. pylori* infection, (iv) the age of the patient at which *H. pylori* is acquired affects the ultimate clinical outcome.

CagA strains were detected in 78.9% of gastric ulcer patients and 56.0% of chronic gastritis patients. The strains from GU and GC patients showed different RFLP pattern (Awakawa et al., 1995). CagE gene and vacA s1 genotype was more frequent in patients with gastroduodenal pathology. The vacA s1 genotype was associated with presence of CagA antibodies. CagE and vacA s1 are more prevalent in patients with peptic ulcer or gastric cancer (Fallone et al., 2000). Tytgat (2000) finds that both cagA and vacA s1 were strongly associated with peptic ulcers and iceA allelic types were independent of cagA and vacA status.

Zheng et al. (2000) indicated that peptic ulcer disease was associated with increased expression of Lewis antigen but not cagA, iceA, and vacA genotypes in *H. pylori* isolates. They have suggested the importance of host-pathogen interaction in the clinical outcomes of *H. pylori* infection. Of the 108 isolates analyzed, the authors observed 95.4% expressed Le^x and/or Le^y, while Le^a and Le^b were expressed in 21.2% and 43.5% respectively. Expression of one or more Lewis antigens (Le^x, Le^y, Le^a, or Le^b) was significantly higher in *H. pylori* isolated from ulcer patients than in non-ulcer patients. They also demonstrated the blood group A antigens on the isolates.

Statistically significant relationships were noted between association of lectin reaction pattern MH4 and atrophic gastritis in European patients. No strain with this pattern was isolated from chronic gastritis. Isolates with pattern MH13 and MH16 was associated with non-ulcer diseases in Swedish population, and all 4 isolates from adenocarcinoma possessed MH16 pattern. From ulcer patients MH1 and MH10 (high lectin reactivities) lectin pattern strains were isolated (Hynes et al., 2002).

GERD was found to be associated with lower rate of *H. pylori* infection, a lower rate of vacA s1 genotype and a higher rate of both vacA s2 and m2 genotypes. This led to the speculation that *H. pylori* infection had a protective effect against GERD (Fallone, 2000). The incidence of new GERD-type symptoms or endoscopic oesophagitis was greater in patients in whom successful *H. pylori* eradication was achieved. Weston (2000) reported a protective effect of *H. pylori* infection on the development of Barrett's adenocarcinoma. Colonization by cagA$^-$ *H. pylori* strains may be protective against the formation of short and long segment Barrett's oesophagus and its malignant complications (Vaezi et al., 2000).

In a study among Tibetan monks in Southern India settlement, it was observed that neither *H. pylori* infection nor gastritis is significantly associated with the dyspepsia (Katelaris et al., 1992).

Patel et al. (1994) raises the possibility of the role of *H. pylori* in delayed or diminished pre-pubertal growth spurt in girls, since they had observed greater reduction of growth among girls who have anti-*H. pylori* IgG in their saliva. Raymond (1994) observed higher prevalence of *H. pylori* infection in children examined because of short-stature.

The results obtained by Tanigawa et al. (2000) suggested that the fishmeal contains factors which greatly enhanced *H. pylori* induced gastritis in Mongolian Gerbils. The identification of the gastritis enhancing factors in the food is relevant since the incidence of gastritis and gastric cancer are very high through out the world.

O'Connor (1995) suggested that *H. pylori* eradication may accelerate ulcer healing. Successful cure of the infection was found to induce a better response in the healing of peptic ulcer: GU 88% vs 73%, DU 95% vs 76%, and unspecified peptic ulcer 95% vs 76% respectively for patients in whom *H. pylori* was cured versus those who remained *H. pylori* positive (Tytgat, 2000). The improvement of gastritis score, healing and recovery from

pain in ulcer patients, and remission of MALT lymphoma provides final proof for the causative role of *H. pylori* infection in various gastroduodenal symptoms.

Gastric cancer

Gastric cancer is one of the most common malignancies in the world. In recent decades the incidence and the mortality rate of gastric cancer is found to be decreasing. Neoplasia is the ultimate result of a disruption of the exquisite mechanisms regulating the normal cell cycle. In addition to proliferation, tissue growth is determined by the balance of cellular differentiation, senescence, and programmed cell death (apoptosis).

Cell progression through the cell cycle is regulated primarily at two points: the G_2/M and G_1/S phase transitions. Regulation appears to be achieved principally by the actions of cyclins and cyclin-dependent kinases. Each cyclin forms a complex with cyclin dependent kinase (cdk) in a cell-cycle dependent fashion, and this complex may result in the acquisition of specific kinase activities. Natural cdk inhibitors also regulate the cell cycle. p^{21} (also called WAF 1 and Cip 1) is a general inhibitor of cdks. p^{21} is transcriptionally activated by the p^{53} tumour suppressor gene product and by cell senescence. p^{53} independent mechanisms also can activate p^{21}.

Peptide growth factors, extracellular matrix and cell-cell adhesion molecules have a significant impact on cell proliferation. In addition, genetic and environmental factors play important role in tumourigenesis. Somatic mutations can result from any class of carcinogens, including chemical mutagens, and ionizing and ultraviolet radiations. Dietary constituents and their metabolites may act as important environmental mutagens within the gastrointestinal tract (GI tract). Viral agents like papilloma virus, EBV, etc. can lead to tumourigenesis in GI tract. All these indicate the multifactorial induction of gastric cancer.

In a review, Hwang et al. (1994) pointed the factors that may enhance gastric cancer risk as the consumption of nitrites, nitrates, alcohol and highly salted, pickled.

fermented or smoked foods. The major environmental factors include *H. pylori* infection, inappropriate food storage, metal and cement dust exposure and cigarette smoking. The gastric cancer risk can be reduced by the high intake of fruits, vegetables, and anti-oxidants such as β-carotene, vitamin B, and vitamin C.

The International Agency for Research on Cancer (IARC) estimates that ⅓ of all gastric carcinomas in the developed countries and ½ in the developing countries were *H. pylori* related. According to the data of Parsonnet et al. (1991), 60% of gastric adenocarcinomas were attributable with *H. pylori* infection. In their nested case-control study the persons seropositive for *H. pylori* were about three times more likely to have gastric adenocarcinoma in the ensuing 1-24 years of follow-up. *H. pylori* can be considered as the missing general worldwide environmental factor in the pathogenesis and epidemiology of gastric cancer.

Most of the studies relating *H. pylori* and gastric cancer are focused on serological prevalence of infection. Forman et al. (1991) reported that between 35 and 55% of all cases of gastric cancer were associated with *H. pylori* infection. In 75% of the gastric carcinoma cases, chronic gastritis is the background lesion (Sipponen et al., 1992). High prevalence of *H. pylori* was observed in a population in Chiapas, Mexico, who were at high risk of gastric cancer (Guarner et al., 1993). The histological analysis showed 85.1% association between *H. pylori* and pre-neoplastic or neoplastic lesions in patients with intestinal metaplasia and 93.8% association in patients with atrophy.

Tsugane et al. (1993), while assessing the level of serum pepsinogen, found that *H. pylori* infection and low level of β-carotene, presumably related to less frequent intake of yellow vegetables, were associated with the risk of atrophic gastritis and this, in turn, is strongly related to gastric cancer risk. A report from Beijing showed the association of *H. pylori* in 32.1% of gastric cancer and the authors suggested the role of *H. pylori* induced

inflammation in the pathogenesis of gastric cancer (Ji et al., 1993). Crabtree et al. (1993) reported 78.5% prevalence while Kuipers et al. (1993) and Nogueira et al. (1993) reported 46% and 82.5% respectively. The Eurogast study group reported an approximately 6 fold increased risk of gastric cancer in populations with 100% *H. pylori* infection compared with populations that have no infection (Eurogast study group, 1993).

Many authors contradicted the role of *H. pylori* in gastric carcinoma. Case-control studies could not find any difference in the prevalence of anti-*H. pylori* antibodies in gastric cancer patients and controls in Portugal (Estevans et al., 1993). Seroprevalence study in Taiwan population reemphasized the association of *H. pylori* in gastric ulcer but not adenocarcinoma (Lin et al., 1993). The prevalence of *H. pylori* positivity and geographical variation of gastric cancer does not correlated in Italian population (Palli et al., 1993). Archimandritis et al. (1993) was not able to find any association between *H. pylori* infection and non-cardia gastric cancer in Greece. Most of these authors considered *H. pylori* as a cofactor in carcinogenesis in population exposed to other carcinogenic factors. According to a Muszynski et al. (1995) *H. pylori* plays only a contributory role in multifactorial etiology of gastric cancer.

H. pylori gastritis is a necessary, though not sufficient cause for many cases of gastric cancer (Goodwin, 1993). Gastric cancer is commonly preceded by atrophic gastritis and intestinal metaplasia. Atrophy occurs after many years of chronic gastritis, which is most commonly due to *H. pylori.* Atrophy leads to hypochlorhydria and low luminal concentration of ascorbic acid, followed by gastric colonization of other bacteria that generate N-nitrosocompounds. At the cellular level, persistent *H. pylori* inflammation induces several mutagenic processes including cell proliferation, possibly by increased production of epidermal growth factors, and DNA damage by neutrophil products.

Serological, histological and PCR study in gastric cancer patients showed a low rate of current and previous *H. pylori* infection. This supported the suggestion that *H. pylori* infection may not be directly associated with gastric cancer (Lin et al., 1994). Takahashi et al. (1993) also reported similar results.

But later, several reports were accumulated which showed the association of *H. pylori* infection in gastric cancer. Most of these reports were based on epidemiological studies. In 1994, IARC (International agency for research on cancer), a subordinate organization of WHO (World Health Organization), identified *H. pylori* as a "group 1 (definite) carcinogen" (Anonymous, 1994). This attracted great interest world wide on the association between *H. pylori* and gastric cancer.

Uemura et al. (2001) observed the gastric cancer development in persons infected with *H. pylori* but not in uninfected persons in their study group. Their data showed an increased risk of development of gastric cancer in those with severe gastric atrophy, corpus predominant gastritis, or intestinal metaplasia. Increased risk is associated with NUD, GU, or gastric hyperplastic polyps also.

Among the *H. pylori* carriers in China a significantly higher gastric cancer rate was observed (Ching and Lam, 1994). Munoz (1994), while reviewing the literature, observed that the studies with a wide range of gastric cancer rates and in which large numbers of populations were compared showed weak but significant positive correlation with *H. pylori* infection and gastric cancer. According to the author, the lack of association between *H. pylori* and gastric cancer reported from high risk countries may be due to the use of inappropriate serological assays. In the countries, where there is higher incidence of *H. pylori* infection, the lack of association may be due to the high prevalence of anti-*H. pylori* antibodies in the general population (Estevans et al., 1993).

The molecular events leading to the pathogenesis of gastric adenocarcinoma is given in the figure 1.3.

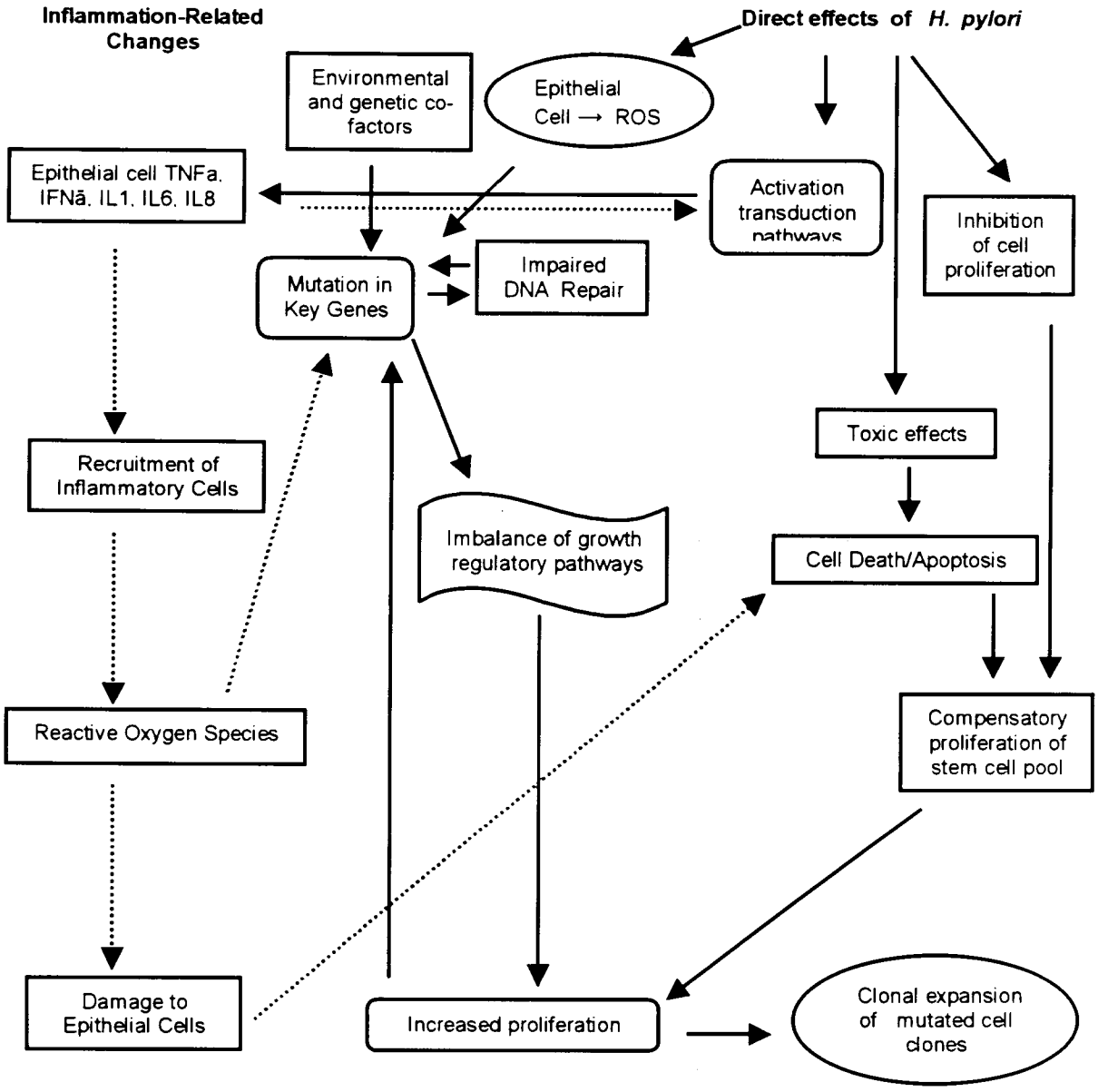

Figure 1.3 Molecular pathways linking *H pylori and* gastric carcinogenesis (Asaka et al., 2001)

Vollmers et al. (1994) isolated two monoclonal antibodies from patients with gastric carcinoma and *H. pylori* associated chronic gastritis. In western blot analysis they showed the recognition of 140 kD and 110 kD bands of analogous tumour extracts and cross reaction with 55 kD and 80 kD bands of *H. pylori* extracts. They have demonstrated the *in vitro* cell proliferation by these antibodies. Because of the simultaneous reaction and stimulation of tumour cells they cause, the authors speculated that these antibodies might be indirectly responsible for the higher incidences of gastric cancer in *H. pylori* positive patients.

The association between non-Hodgkins lymphoma and *H. pylori* infection was reported (Parsonnet et al., 1994). Ashorn et. al. (1994) successfully treated a child with non-Hodgkins lymphoma and gastritis caused by *H. pylori* with antibiotics and chemotherapy and suggested the eradication of *H. pylori* infection for regression of non-Hodgkins lymphoma.

Several authors reported the crucial role of *H. pylori* infection in follicle development and progression to lymphomagenesis (Eidit et al., 1994; Dixon, 2001). Hussel et al. (1993) reported the strain specificity of *H. pylori* to stimulate cellular proliferation in low-grade B-MALT lymphoma. According to Fagioli et al. (1994) *H. pylori* infection could be considered as a pathogenic factor in 34% MALT lymphoma patients. The cure of *H. pylori* infection led to the remission of MALT (Nakamura et al., 2000). Based on the response to eradication of *H. pylori* these authors classified MALT lymphoma into 3 types: MALT A. MALT B, and MALT C. They speculated that MALT A represents an incipient neoplasm or dysplasia. MALT B a neoplasm activated by antigenic stimulation of *H. pylori* and MALT C a lymphoma independent of *H. pylori.*

Atrophic gastritis (or intestinal metaplasia) and gastric cancer are very much alike in time trends and in course. This parallelism favours the suggestion that *H. pylori* gastritis

with atrophic and metaplastic sequelae (atrophic gastritis) contribute to the pathogenesis of gastric cancer (Sipponen and Kimora, 1994). In a case-control study, Sipponen et al. (1994) reported slightly but significantly increased risk of gastric carcinoma in patients with non-atrophic gastritis. The risk of diffuse type gastric carcinoma was higher in non-atrophic gastritis than the risk of intestinal type, where as the opposite was the case in patients with atrophic gastritis. Murakita et al. (1994) and Hirai et al. (1994) reported the strong association of vacuolating cytotoxin producing *H. pylori* with gastric carcinoma.

Several studies showed the contribution of *H. pylori* infection in the progression of superficial gastritis to atrophic gastritis and intestinal metaplasia. Kuipers et al. (1995) studied *H. pylori* positive and negative patients for a period of 10-13 years and found that 28% of positive patients showed the progression of gastritis to atrophic gastritis and metaplasia while in negative patients only 2% subjects showed such progression. Sakaki (1995) also reported similar results. In their study 22% of positive patients showed various degrees of expansion of atrophic area and during their follow-up, 4 patients developed well-differentiated mucosal cancer.

Genta (1995) in a review gave the sequence of events leading to the development of carcinoma as chronic active gastritis→atrophic gastritis→Intestinal metaplasia→ dysplasia→neoplasm. Asaka et al. (1995) reported a strong association of *H. pylori* infection with intestinal metaplasia and gastric carcinoma in Japanese population and they suggested that the *H. pylori* infection leads to the development of gastric cancer by causing severe atrophic gastritis.

Relatives of patients with noncardia gastric cancer have an increased prevalence of hypochlorhydria and atrophy and that these pre-cancerous abnormalities are almost entirely confined to subjects with *H. pylori* infection (El-Omar et al., 2000). The presence of intestinal metaplasia was significantly more frequent in cancer patients than in controls

(Shimoyama et al., 2000). In another report Shimoyama et al. (2000) pointed that severe expression of corpus gastritis does not seem to be specific characteristic of patients with gastric cancer. In population with high risk of gastric cancer, the extension of intestinal metaplasia correlates with the extent of its "incomplete phenotype" and is significantly associated with increased cancer risk (Cassaro et al., 2000).

The findings of Blaser et al. (1995) indicated that the infection with *H. pylori* at a very young age may particularly vulnerable period and could lead to increased cancer risk. In their study men from larger families who were *H. pylori* positive was associated with more than twice the risk of developing gastric cancer than being part of a smaller family. The possible mechanisms through which *H. pylori* exert pathogenic effects are continuous inflammation in adulthood and/or irreversible damage to gastric mucosa in childhood or teenage years (Kikuchi et al., 2000). Brenner et al. (2000) reported the higher prevalence of *H. pylori* infection among subjects with a parental history of stomach cancer than among other subjects. Thus, in familial aggregation of stomach cancer *H. pylori* infection have a role besides other factors.

The role of *H. pylori* infection in gastric carcinogensis was studied at cellular and molecular level. Lynch et al. (1995b) reported the increased cell proliferation in *H. pylori* gastritis. They observed a synergistic effect of *H. pylori* infection and bile on gastric epithelial cell proliferation. So it was speculated that *H. pylori* damages the gastric mucosa and this lead to compensatory increase in mucosal proliferation, which may expose vulnerable cells to luminal mutagens. Only in *H. pylori* associated gastritis, but not in chronic gastritis where the organism is absent, the gastric cell proliferation was increased (Lynch et al., 1995a). After triple therapy to gastritis, normal mucosal proliferation rate was observed in those subjects in whom the eradication was successful, but those in whom eradication failed, cell proliferation returned to its previously high level. Thus it was suggested that *H. pylori* infection might be playing a part in gastric carcinogenesis by

increasing the gastric cell proliferation. Cytokines, free oxygen radicals, and other factors released by the inflammatory response following infection interact with cells in the proliferative compartment to increase cell proliferation (Lynch and Axon, 1995). Due to atrophy and intestinal metaplasia, hypochlorhydria occurs which leads to the proliferation of bacteria capable of nitrate reduction with consequent production of nitrosocompounds, which in turn may be responsible for mutagenic events.

The distribution of proliferating cells is different in the case of gastritis compared to that observed in normal gastric tissue. With this finding, Panella et al. (1996) suggested that in response to *H. pylori* infection, cells outside the G_0 phase of cellular cycle replace mucus producing columnar epithelium. Some of these cells may be immature and contain a small amount of neutral mucins, ineffective in protecting the epithelial barrier. In this condition, the action of other aggressive luminal factors may be enhanced and progression of *H. pylori* damage occurs. In intestinal metaplasia this event is maximally expressed and the persistence of *H. pylori* becomes associated with a high rate of cell division.

In vitro studies with various mammalian cell lines demonstrated the anti-proliferative activity of *H. pylori.* The proliferation inhibiting protein (PIP) of *H. pylori* is reported to be a 100 kD factor (Knipp et al., 1996; Smoot et al., 1999). Flow-cytometric analysis showed S-phase accumulation of HGC-27 cells, and suggested that ammonia inhibited the growth of gastric cells in S-phase (Matsui et al., 1995). This mechanism could contribute to *H. pylori* associated mucosal atrophy, a known risk factor for gastric cancer.

Matsui et al. (1995) demonstrated the alteration of cell cycle phase distribution in HGC-27 cell lines at an ammonia concentration of ≥ 0.04%. *In vivo* ammonium chloride is generated from a combination of ammonia with HCl of parietal cell origin. Gastric juice ammonia in *H. pylori* infected persons is 34±16 mM (Marshall and Langton, 1986).

Anti et al. (1998) observed a significantly enhanced proliferation within the gastric epithelium of *H. pylori* positive subjects and this was positively correlated with both acute and chronic inflammatory cell densities. Progressively increasing rates of proliferation and apoptosis in epithelial cells is a characteristic feature of *H. pylori* gastritis, whereas the development of intestinal metaplasia is characterized by much lower rate of apoptosis along with relatively increased proliferation (Scotiniotis et al., 2000). These changes, possibly irreversible, may represent a crucial step in the gastric carcinogenesis resulting from *H. pylori* infection. El-Zimaity et al. (2000) observed increased proliferation in presence of *H. pylori* and after successful therapy the rate of proliferation in the corpus declined while that in antrum remained increased even after 1-3 year follow-up. Watanabe et al. (2000) demonstrated the *H. pylori* induced apoptosis of MKN 45 cells in the presence of inflammatory cytokines IFN-γ and TNF-α. Suzuki and Ishi (2000) reviewed apoptosis as well as its associated pathological events; such as hyper proliferation in *H. pylori* colonized gastric mucosa. Kim et al. (2000) reported the enhanced apoptosis of the infected epithelial cells in presence of TNF-α and soluble Fas L produced as part of immune response by migrating neutrophils. This gave the evidence for the interaction of *H. pylori* infected epithelial cells with neutrophils in the process of apoptosis. *H. pylori* directly induced apoptosis mainly through cytochrome C release from the mitochondria (Maeda et al., 2002). *H. pylori* also exerted anti-apoptotic effects through Nf-κB activation. Both these effects were dependent on Cag PAI.

The vacuolating cytotoxin producing strains were strongly associated with both atrophic gastritis and gastric carcinoma (Murakita et al., 1994). Hirai et al. (1994) also reported the same and demonstrated that cytotoxin neutralizing sera from gastric cancer patients recognized an 87 kD band in *H. pylori* extract. Klaamas et al. (1996) could not find any difference in *H. pylori* seroprevalence between the intestinal and diffuse type gastric

cancer patients. The response related to VacA and CagA were also not changed and was observed more often in the younger group of cancer patients.

70.6% of isolates from gastric cancer showed vacA s1/m1 genotype and cytotoxicity and 88.2% were cagA positive and this was significantly higher when compared with control groups (Miehlke et al., 2000). The authors concluded that in addition to determinants of bacterial virulence, environmental and host factors such as the distribution of *H. pylori* gastritis were likely to contribute to the development of gastric carcinoma.

By analyzing various studies related to the presence or absence of various oxidants and antioxidants in *H. pylori* infections, Correa (2000) hypothesizes that the lower concentrations of antioxidants in gastric mucosa facilitates gland lose and are indicators of multifocal atrophy. The interplay of oxidants and antioxidants in carcinogenesis is a challenge to scientific community and in the context of *H. pylori* infection and gastric cancer this becomes more significant since the concentrations of vitamin C in gastric juice of *H. pylori* positive patients is reported to be lower. Rokkas et al. (1995) reported that eradication of *H. pylori* restores gastric juice vitamin C levels and there by improve antioxidant defenses. Ascorbic acid is acting as a scavenger of free radicals generated in human gastric mucosa. Free radical scavenging role of ascorbic acid results in the generation of ascorbyl radical in gastric mucosa and experimentally determined concentration of this radical are greater in a group of patients at increased risk of gastric cancer (Drake et al., 1996). Davies et al. (1993) have reported an increase in oxygen radical production in both the duodenal and gastric pyloric mucosa after infection with *H. pylori.* Patients infected with *H. pylori* expressed more inducible nitric oxide (iNOS) and CagA positive strains caused higher levels of iNOS (Li et al., 2003). Tatsuguchi et al. (2000) detected cox-2 mRNA and protein in gastric ulcer tissues but not in intact mucosa. The percentage of cox-2 expressing cells was higher in open than in closed ulcers, and in

gastritis than in gastric mucosa without *H. pylori* infection. Both nitric oxide and cox-2 products have mutagenic potential.

With increased cell turn over of gastric mucosa during active *H. pylori* infection, an increased load of mutations may occur as a consequence of the infection and other environmental risk factors (Asaka et al., 2001). Microsatellite instability (MSI) is a marker of mutations that develop subsequent to deficient DNA mismatch repair (MMR) activity. Some studies revealed that patients with MSI positive tumours showed a significantly higher frequency of previous *H. pylori* infection (Wu et al., 1998). Recently active *H. pylori* infection were reported in patients with MSI-positive tumours (Leung et. al., 2000)

Kim et al. (2002) suggested that the *H. pylori* infection might lead to deficiency of DNA MMR in gastric epithelial cells. This may increase the risk of gastric cancer during the chronic *H. pylori* infection. Impairment of DNA MMR system represents a novel mechanism of infection associated cancer promotion.

H. pylori exert inhibitory effect on cell cycle progression *in vitro* and this occurs predominantly at the G_2-G_1 check- point. Since KATO III cells, which have a deleted p^{53} gene, showed no response to *H. pylori* suggested that p^{53} may be important in the *H. pylori* induced cell cycle arrest. Results also supported the role for cyclin-dependent kinase inhibitors in the G_1 cell cycle arrest exerted by *H. pylori* and its involvement in changing the resulting proteins p^{53}, p^{21}, and cyclin E in the cell cycle. *H. pylori* may exert its effect on cell cycle regulatory proteins by increasing p^{53} expression leading to an increase in p^{21} expression., resulting in cell cycle arrest or by a down regulating expression of cyclin E. The cell cycle arrest then causes the adjascent undamaged cells to increase in overall cell proliferation seen *in vivo*. As an agent that induces cell stress, *H. pylori* may reduce the ability of damaged cells *in vivo* to repair significant cell injury, and may increase the chance of genomic instability and cancer (Ahmed et al., 2000).

The association with *H. pylori* infection is similar regardless of the histological features of the tumour, whereas the association is stronger for non-cardiac gastric cancer than for cardiac gastric cancer (Hansson et al., 1995).

The association of *H. pylori* infection in the development of MALT lymphoma has been established (Tursi et al., 1997; Hussel et al., 1993). Several authors reported the regression of MALT lymphoma by *H. pylori* eradication (Nakamura et al., 2000; Roggero et al., 1995). Calvert et al. (1995) reported the genetic changes occurred during the Helicobacter-associated transition from chronic gastritis to MALToma. Wotherspoon et al. (1993) suggested anti-*H. pylori* treatment for lymphoma patients.

Several animal model studies were conducted to generate direct evidence of the causal relationship between *H. pylori* infection and occurrence of gastric cancer. Hirayama et al. reported first in 1996 that *H. pylori* could induce gastritis, gastric ulceration, and intestinal metaplasia during long-term *H. pylori* infection in Mongolian Gerbils. In their model *H. pylori* colonized the stomach and induced gastritis 12 weeks after inoculation and ulceration developed at 24 weeks and intestinal metaplasia at 24-48 weeks. The histological changes occurred were similar to those in human infection. Watanabe et al. (1998) reported that long-term infection with *H. pylori* alone could induce gastric adenocarcinoma in Mongolian Gerbils. In their study 37% of infected Mongolian Gerbils developed gastric cancers, and all of them were well-differentiated, intestinal type carcinoma.

Shimuzu et al. (1999) reported that *H. pylori* infection plus administration of MNNG (N-methyl-N-nitro-N'-nitrosoguanidine), a chemical carcinogen, enhanced the development of gastric cancer. Fujioka et al. (2000) also reported similar results with MNNG and MNU (N-methyl-N-nitrosourea). The authors also developed gastric cancer in Mongolian Gerbils with *H. pylori* infection alone

In relation to cancer prevention, Shimuzu et al. (1999) reported that *H. pylori* eradication could decrease the incidence of gastric carcinoma in Mongolian Gerbils induced by *H. pylori* inoculation plus administration of low-dose chemical carcinogens. Eradication of *H. pylori* in the early phase of the process of carcinogenesis reduced the occurrence of gastric cancer in animal models. These experimental models provided conclusive evidence to the etiologic role of *H. pylori* in gastric cancer development.

From these facts, it can be presumed that *H. pylori* might be causing gastric carcinomas by converging effects of two main types of events: (i) collateral damage of inflammatory by-products causing mutational events in gastric epithelial cells, and (ii) direct effects on gastric epithelial cells by *H. pylori* organism or released bacterial products at different levels.

Gastric cancer is an example of a cancer associated with a chronic inflammatory process that results in a metaplastic epithelium and cancer (Graham, 2000). In general the risk is related to the extent and severity of the atrophic changes present. Because *H. pylori* infection causes the underlying gastritis, it is the critical variable. Histological studies have shown that gastric cancer without underlying gastritis is rare. All the problems regarding making causal association between viruses and cancer are applicable to *H. pylori* and gastric cancer, including (i) there is a long incubation or induction between infection and cancer, (ii) the candidate agent is common but cancer is relatively rare, (iii) there is need for cofactors, (iv) the cause of cancer may vary depending on the geographical area or age, (v) different strains may have different oncogenic potential, (vi) the human host plays a critical role in susceptibility, (vii) cancer is multifactorial and the agent may play roles at different points, and (viii) there are often other causes of cancer. Single nucleotide polymorphisms within the promoter region of several critical proinflammatory genes dramatically increase the risk of *Helicobacter*-associated gastric cancer (Li et al., 2003)

The incidence of gastric cancer varies in different regions and can fall rapidly, even in the same population in relation to the levels of sanitation, standards of living, food storage and use of salts, changes in diet with fresh fruits and vegetables, and a fall in the incidence of febrile childhood disease (Graham, 1997). Since *H. pylori* and gastric cancer are a public health problem, the measures could and should be taken to cancer prevention including

1. Education into hygiene, which could help to prevent person-to person bacterial spread.
2. Population based eradication therapy or anti-*H. pylori* vaccination (Goldstone et al., 1996). Elimination of *H. pylori* will make gastric cancer a rare disease.

Immunity to infection

Relatively impervious mucosal barrier, salivary lysozyme, gastric acid, pancreatic digestive enzymes, and detergent effects of bile acids constitute the major innate defense mechanisms of gastro intestinal tract. Bacterial species that evade these mechanisms but do not adhere to or invade epithelial cells are removed by intestinal secretion and peristalsis. The antibacterial peptides secreted by Paneth and epithelial cells further limits bacterial colonization. Mucus layer shields the epithelium from luminal bacteria and epithelial tight junctions exclude the macromolecules.

Between 60% and 80% of the body's immunoglobulin secreting cells are found within mucosal surfaces, and most are present in gastric associated lymphoid tissue (GALT). Mucosal lymphocytes stimulated in any one region can return to other mucosal surfaces, laying the foundation for a common MALT in which antigen stimulation at any site (nasal, oral, rectal, inhaled) can generate protective response at all mucosal surfaces.

The high prevalence of *H. pylori* infection in adults through out the world shows that the natural mechanisms of resistance are apparently insufficient to protect the individuals. But the fact that some individuals remain free of the infection during their lifespan even in

areas with a very high prevalence of *H. pylori* infection indicates that a natural resistance to *H. pylori* does exist.

The inflammation associated with *H. pylori* infection is characterized by infiltration of polymorph nuclear cells and mononuclear cells into the lamina propria (Blanchard et al., 2000). IgA plasma cells, B-cells and Th cells constitute the infiltrated mononuclear cells (Hartz et al., 1996).

Rossi et al. (2000) demonstrated the recruitment of polymorphonuclear cells at early stages and later mononuclear cells into the gastric mucosae of Beagle dogs experimentally infected with *H. pylori*. Organization of infiltrated cells resulted in the development of lymphoid follicles, which became evident macroscopically. Degranulation of neutrophils occurs in a concentration dependent manner by direct stimulation with *H. pylori* sonicate proteins (Noorgaard et al., 1995) and increased neutrophil oxidative burst stimulated by *H. pylori* is thought to play a major role in enhanced gastric mucosal damage and consequent atrophic gastritis and gastric cancer (Mizuki et al., 2000).

Adhesion of *H. pylori* to the epithelium induces the translocation of antigens into epithelial cells. This results in the activation of Nf-κB transcription factor and production of inflammatory cytokines (Keates et al., 1997). Elevated levels of IL1β, IL-6, IL-8, IL-12, TNF-α, growth related oncogenes, monocytic chemotactic protein-1, macrophage inflammatory protein-1α, and RANTES chemokines were found to be elevated in gastric biopsies from infected subjects (Reviewed by Ibrahimov and Pappo, 2000). The influence of these cytokines and chemokines lead to the activation of immune mechanisms that ordinarily lie dormant in gastric tissue. Thus the infected stomach function as a target end organ for leukocyte homing and amplifies the immunologic machineries to potentiate local T-cell activation (Pappo et al., 2001).

H. pylori infection induced strong antibody responses in the human gastric mucosa both in asymptomatic carriers and in duodenal ulcer patients (Mattsson et al., 1998). *H. pylori* elicit both local and systemic responses in the host. This response includes an elevation in specific antibody of the serum IgG and IgA classes as well as sIgA and low levels of gastric IgM (Rathbone et al., 1986). The findings of Liutu et al. (2004) reported increased IgE production in gastritis patients with *H. pylori* infection.

Proportion of IgG present in gastric cancer patient sera is directed to Le^x determinants of *H. pylori*. Le^x has an ability to mimic a human oncofoetal antigen. This not only may assist in colonization and long-term infection but also may augment the development of ulcers and gastric carcinoma (Sherbume and Taylor, 1995). But Shimoyama et al. (2000) suggested that serum ant-Le^x antibody does not play a primary role in the development of gastric cancer in patients with *H. pylori* infection. The positivity rate of tissue IgA against *H. pylori* were lower in the mucosa of intestinal metaplasia than in non-metaplastic gastric mucosa and were negative in gastric carcinoma (Matsukura et al., 1995). The same authors reported the positivity and negativity of serum IgA and IgG antibodies against *H. pylori* coincided with the presence or absence of tissue IgA antibody in the non-metaplastic gastric mucosa. The seroprevalence of *H. pylori* infection in gastric cancer patients was high in Italian patients. An oligoclonal response was more common in gastric cancer compared with non-gastric cancer and outpatients (Menagatti et al., 1996).

All chronic gastritis patients showed mucosal IgA to *H. pylori* (Crabtree et al., 1992). In patients, the positivity and negativity of serum IgA and IgG antibodies against *H. pylori* coincided with presence or absence of tissue IgA antibody in non-metaplastic gastric mucosa (Matsukura et al., 1995).

The initial antibody response against *H. pylori* showed variability in children and adult patients. Antibody response was predominantly against small molecular weight

antigens in children while in adults it is against larger molecular weight antigens (Mitchell et al., 1996). Kindermann et al. (2001) speculated that young children may have a different immune response to *H. pylori* infection and may respond to certain *H. pylori* antigens only.

On immunoblotting *H. pylori* seropositive sera recognized nine major bands with molecular weights 120 kD, 85 kD, 66 kD, 62 kD, 59 kD, 47 kD, 43 kD, 33 kD, and 28 kD (Klaamas et al., 1996). The comparison of antigenic patterns recognized by *H. pylori* seropositive sera from patients having different gastroduodenal pathologies revealed no association of specific *H. pylori* antigens with antibodies (Kimmel et al., 2000). A significant positive correlation between *H. pylori* IgG antibody titres and patient age were observed (Crabtree et al., 1991).

In gastric cancer patients, antigen recognition pattern by anti-*H. pylori* systemic IgG response was comparatively homogeneous while mucosal IgA response showed considerable heterogeneity (Crabtree et al., 1993). Menegatti et al. (1996) reported oligoclonal response as more common humoral response in gastric cancer compared with both non-gastric cancer and out patients.

Mucosal immune response may not be sufficient or even necessary for protection against *H. pylori* infection. There are no reports showing increased frequency of *H. pylori* infection in IgA deficient individuals compared to subjects with normal IgA levels. Studies in mice have showed that immunity to *H. pylori* can be induced in the absence of B-cells (Blanchard et al., 1999).

In the stomach tissue there are not many T-cells. Those T-cells, which are present in the stomach tissue appear to be biased toward the Th1 phenotype (Haeberle et al., 1997). Karttunen et al. (1995) reported a Th1 pattern of lymphocyte activation in gastritis irrespective of *H. pylori* status. Populations of gastric T-cells analyzed from *H. pylori* infected biopsies showed an overabundance of IFN-γ^+ and IFN-γ secreting cells

(Bamford et al., 1998). This cytokine profile is in accordance with the abundance of Th1 phenotype. The transendothelial migration of Th1 cells was mediated by RANTES produced by endothelial cells (Kawai et al., 1999). Taken together it can be assumed that not only *H. pylori* infection but also the repertoire of cytokines in the stomach tissue causes the predominance of proinflammatory Th1 cells in the infected stomach. In addition to any effects *H. pylori* may have on the selection of Th1 cells, this species of bacteria may also have chosen a niche in which the host response is already biased toward Th1 response that cannot orchestrate the clearance of the infection.

Alkout et al. (2000) reported a significant correlation between *H. pylori* binding and proinflammatory cytokine production by human leukocytes. They observed higher levels of proinflammatory cytokine production by leukocytes of patients who developed peptic ulcer disease than those of patients with normal endoscopy. Thanahashi et al. (2000) demonstrated the induction of proinflammatory cytokines in response to purified *H. pylori* urease by MKN-45 cells *in vitro*.

Immunization of mice with *H. pylori* lysate, VacA and urease showed that bacterial lysate provided almost complete protection of mice from infection while VacA protected mice from infection with Type I strains. Urease also protected mice (Marchetti et al., 1995). Oro-gastric vaccination with recombinant *H. pylori* catalase plus cholera toxin protected a significant proportion of mice from infection (Radcliff et al., 1997).

Therapeutic oral vaccination with *H. pylori* whole cell sonicate and cholera toxin caused eradication of bacteria from 62.5% of mice persistently infected with *H. pylori* (Ikewaki et al., 2000). The efficacy of vaccination depended on the mucosal IgA response in the gastrointestinal tract against *H. pylori* via Th2 cell activation. In intragastric immunization against *H. pylori* saliva is necessary for the induction and maintenance of optimal immunity in the stomach (Shirai et al., 2000).

Recently Arcila et al. (2002) developed recombinant vaccine strains of *Salmonella enterica* serovar *Typhi* capable of expressing *H. pylori* urease and demonstrated its effectiveness for immunization of mice against *H. pylori* infection. Intranasal administration of vaccine resulted in induction of anti-urease immune response skewed toward Th1 phenotype. Following subcutaneous booster dose with urease plus alum enhanced immune response and led them to more balanced Th1/Th2 phenotype.

Markers of Infection

One biochemical marker for an active *H. pylori* infection is an increase in serum gastrin levels (Graham et al., 1990; Collins, 1992; Murakami et al., 1995). Elevated serum pepsinogen was related to the presence of *H. pylori* infection (Asaka, 1992) and a decrease of PG I/PG II was more frequent among *H. pylori* positive subjects than among negative subjects (Kikuchi et al., 2000). The marked or extreme hypergastrinemia is not specific for *H. pylori* infection, because it is also present in autoimmune gastritis (Sipponen et al. 1990).

Histology provides the marked evidence for *H. pylori* infection. In many cases the presence of *H. pylori* infection is an important marker for inflammatory gastroduodenal disorders (Blaser, 1990). The typical histology is infiltration with a combination of acute and chronic inflammatory cells along with development of intramucosal lymphoid aggregates and follicles (Ota and Genta, 1997). *H. pylori* cells are present in sufficient quantities to be seen with high-power oil immersion magnification of histologic sections or of Gram stains of smears of gastric mucus. Several staining techniques were devised for easy identification of *H. pylori* and certain laboratories Genta or El-Zimaity stains are preferred (Graham and Qureshi, 2001). Since *H. pylori* does not colonize all areas of stomach, Misra et al. (2000) reported that two biopsies taken from A_3 region of stomach are sufficient for

confirmation of presence of *H. pylori* and to get maximum yield of the organism. Thus, the fallacious results due to sampling error can be reduced.

In the beginning itself, it was noticed that the organism produces large amount of urease (Langenberg et al., 1984). Using urease as the marker, several tests - both invasive and non-invasive, were devised for easy and early detection of *H. pylori* infection. Urea breath test (UBT) is the non-invasive method of choice to determine *H. pylori* status either pre-or post-therapy. The test depends on the action of urease on labeled urea administered orally into the person, and the consequent liberation of labelled CO_2 which can be easily detected in the breath. The urea can be labelled with either non-radioactive isotope ^{13}C or radioactive isotope ^{14}C. Accordingly ^{13}C-UBT and ^{14}C-UBT are available commercially. The ^{13}C-UBT has been proven to be extremely reliable test and yields satisfactory results. ^{13}C-UBT gives reliable information about *H. pylori* status before or after therapy (Klein and Graham, 1993; Klein et al., 1996) the ^{14}C-UBT does not reflect *H. pylori* density on histology in patients of DU disease (Kumar et al., 2001).

The observation of the presence of preformed urease produced by *H. pylori* in the biopsy specimens led to device urea broth tests. When a biopsy specimen containing *H. pylori* is introduced into a medium containing urea, urease splits the urea into ammonia and CO_2. The ammonia released results in an increase in pH, which can be detected by the colour change of pH indicator. A modified urea broth test, Rapid urease test (RUT), was devised by McNulty et al. (1989) and was widely accepted. The test showed almost 100% specificity when compared with detection of *H. pylori* by gram staining, culture and histology. Ng et al. (2000) reported that unbuffered RUT, which was frozen at $^{-}20^{\circ}C$ for 4 months, remained highly sensitive in the detection of *H. pylori* infection. RUT is considered to be very simple, sensitive, and specific test which enables the endoscopists to diagnose *H. pylori* infection (Kawanishi et al., 1995). A modified RUT, which provides results in 20

min, has been available on commercial basis. The main drawback of this test is the false negative results in mild infections (Tokunaga et al., 2000).

Anti-*H. pylori* antibody detection is the easiest non-invasive approach to test for the presence of *H. pylori* infection. ELISA tests which uses purified antigens such as high molecular weight antigens (HM-CAP ELISA) and those uses more complex antigens are available (Evans Jr. et al., 1989; Meijer et al., 1997). All these tests are proven to be accurate worldwide. *H. pylori* infection would be missed in most of the patients with atrophic gastritis without analysis of *H. pylori* antibodies (Kokkola et al., 2000).

Several modifications were made in the detection of anti-*H. pylori* antibodies such as FMIA (flow cytometric immunofluorescence assay) (Best et al., 1992), rapid flow through membrane based EIA (Pronovost et al., 1994), dry latex agglutination test (Midolo et al., 1995). Recently, Atanassov et al. (2002) proposed two new antigens – β-keto-acyl-ACPS and PPiase (Propyl-cis-trans-isomerase) as antigens of diagnostic interest.

Although tests for IgA and IgM to *H. pylori* are available, the US FDA approved none. Neither IgM, nor IgA tests alone are superior to IgG serology, and the sensitivity and specificity of the tests have generally been too low (Graham and Qureshi, 2001). The data obtained by Jaskowsky et al. (1997) showed that the frequency of IgA positive IgG negative patients with gastrointestinal disorders suggestive of *H. pylori* infection is 7.2% only.

H. pylori stool antigen (HpSA) testing is another non-invasive test developed and found to be reliable and easy-to-use test for diagnosing *H. pylori* infection (Vaira et al., 1999). Forne et al. (2000) found that HpSA test using a cut-off value of 0.130 may be useful for the primary diagnosis of *H. pylori* infection with a sensitivity similar to that obtained with other standard tests.

According to Lin et al. (1992) biopsy of the antrum at gastroscopy and RUT are most useful for the initial diagnosis of the infection and for assessing epidemiology and mode of transmission serology is most suitable. Mendall et al. (1995) suggested serology as the method of choice of screening before direct access upper gastro-intestinal endoscopy in those less than 45 years.

Bermejo et al. (2000) successfully used serology for monitoring *H. pylori* eradication 6months after therapy completion. They pointed that loss of antibodies to *H. pylori* is a slow process and that a high percentage of patients successfully treated are likely to exhibit detectable antibody levels for years. Midolo et al. (2000) suggested UBT as the most appropriate test to monitoring the successful eradication, and IgG EIA may useful where breath tests are not available or when the patient remains on proton pump inhibitor. *H. pylori* eradication may be confirmed by using a combination of HpSA and UBT (Ohkura et al., 2000). For evaluating eradication therapy repeat testing should be performed a minimum of 4 weeks after ceasing administration of antibiotics and bismuth preparations. Proton pump inhibitors should also be avoided before testing. In the case of endoscopy-based tests, a minimum of two tests should be done with biopsies taken from both antrum and body (Katelaris and Jones, 1997).

The isolation of organism in culture is 100% specific. This allows testing for antibiotic susceptibility. The sensitivity of the culture varies from 50-99% depending on the laboratory and interest of the microbiologist (Graham and Qureshi, 2001).

PCR techniques are valuable for diagnosis of *H. pylori* infection. Under ideal circumstances the sensitivity of the method is close to that of culture. In a comparative study, van Doorn et al. (2000) observed that PCR had highest diagnostic sensitivity followed by histology, culture and CLO test. Kobayashi et al. (2002) used TaqMan (Real time) PCR to estimate the total number of *H. pylori* genomes in the biopsy samples and

found that the technique had highest sensitivity and specificity when compared to other four tests- UBT, CLO test, culture, endoscopy findings. At the present time PCR remains a research tool for the diagnosis of *H. pylori* infection. There are significant biological differences between distinct genotypic variants of *H. pylori* and these differences may have implications for the effective and reliable detection of this important human pathogen (van Doorn et al., 2000).

Treatment against Infection

Since *H. pylori* infection results in various clinical outcomes, the question "who should be treated" became significant. According to Blaser (1992) the patients with refractory peptic ulcer disease in whom gastric cancer had been ruled out and patients with refractory NUD can be subjected to antimicrobial therapy. He also speculates that establishing a screening programme in areas with high risk of gastric cancer and treating persons who have significant risk factors may reduce the mortality associated with gastric cancer.

In February 1994 at U.S National Institute of Health (NIH)- sponsored consensus conference, an independent panel declared the ulcer as an infectious disease and recommended the therapy to switch from treating symptoms with antacids to antibiotic eradication of the infection (NIH consensus statement, 1994). Telford et al. (1994) indicated the importance of prophylactic vaccination and suggested genetically detoxified. but antigenically intact CagA as an obligate candidate of vaccine.

In vitro, *H. pylori* is naturally resistant to a limited number of antibiotics such as vancomycin, trimethoprim sufonamides and some strains shows natural resistance to β-lactams (Cefsulodin), polymyxin and antifungal compounds. Most of the antimicrobial agents have systemic effects. The activity of antimicrobial agent *in vivo* to *H. pylori* will be

dependent on the pH of the stomach and the agent must reach high mucosal concentrations.

Study of susceptibility of *H. pylori* to various antimicrobial agents showed ampicillin as the most active agent (Rubinstein et al., 1994). In the above study all strains showed susceptibility to tetracycline and all the cephalosporins tested. The pre-treatment resistance and acquired metronidazole resistance may be the factor leading to the treatment failure (Xia et al., 1994). Metronidazole resistance is observed worldwide. So it was suggested to test *H. pylori* susceptibility to metronidazole and interpretation of the results before commencing antimicrobial therapy.

A European study using same methodology in different countries showed an overall resistance of metronidazole 26% which ranged from 7% in Spain to 49% in Greece (Glupczynski and European Multicentre study group on antibiotic susceptibility to *H. pylori*. 1992). The metronidazole resistance in France was 25% but it increased rapidly reaching 50% in 1994 (Megraud et al., 1994). In India over two thirds of all strains from Lucknow and Mumbai were metronidazole resistant (Abraham et al., 1997). About 90% strains from Calcutta were metronidazole resistant (Mukhopadhyay et al., 2000). A report from Netherlands showed a primary resistance to metronidazole in 41% of clinical isolates (Noach et al., 1994).

In general European women harbour more metronidazole resistant strains than men, and this may probably due to the fact that women are more likely to have been treated with nitroimidazole for the gynaecological infection (Megraud et al., 1995). A variety of alterations including inactivation of rdxA gene are frequently associated with *H. pylori* resistance to metronidazole (Tankovic et al., 2000). There are reports showing null mutations in fdxB (feredoxin-like protein), frxA (NAD(P)H Flavin oxidoreductase) or rdxA gene in metronidazole resistance (Kwon et al., 2000; Solca et al., 2000).

The primary resistance to macrolides is much lower than that for nitroimidazole. Westblom and Unge (1992) observed less than 1% macrolide resistance in all the countries where they have tested. Clarithromycin was reported to be most effective in treatment (Graham, 1995). Clarithromycin resistance of *H. pylori* is associated with one of three point mutations in domain v of the 23S rRNA gene: A2143G, A2144G, A2143C (Debets-Ossenkopp et al., 1996; Versalovic et al., 1996; Ochialini et al., 1997). Several tests were developed to test the *H. pylori* resistance to clarithromycin by molecular techniques (Maeda et al., 2000; Trebesius et al., 2000). In North Eastern part of Germany. Wolle et al. (2002) observed an overall primary resistance rate of 26.2% for metronidazole and 2.25% for clarithromycin. Resistance to tetracycline and ampicillin were not observed.

No primary resistance reported to rifamycins in *H. pylori.* But acquired resistance was reported and one study showed a novel codon 149 (v 149 F) in rpoB gene (Heep et al., 2000). The equivalent mutation (v176 F) was reported in several resistant isolates of *Mycobacterium tuberculosis*, another agent causing chronic infection in humans. Moore et al. (1995) reported *H. pylori* resistance to quinolones primarily as a result of alterations in gyrA gene.

There are reports showing the presence of *H. pylori* strains in PU and gastritis patients with both metronidazole resistant and sensitive strains (Loivukene et al., 2000). All these strains were found to be clarithromycin sensitive. Among metronidazole resistant strains combined resistance to clarithromycin, ciprofloxacin, and tetracycline was present (Boyanova et al., 2000). The same study showed some strains with triple resistance to macrolides, metronidazole and either ciprofloxacin or tetracycline. van der Ende et al. (2001) showed the coexistence of clarithromycin resistant and sensitive strains with identical genotypes in patients prior treatment.

In the light of primary resistance and acquired resistance observed in *H. pylori*, it was suggested to choose multidrug therapy instead of monotherapy for effective eradication of the infection. Many studies evaluated the efficacy of the different combinations of drugs. By using Amoxycillin and metronidazole for 1 week and plaunotol for 4 weeks, Karita et al. (1995) achieved 86% eradication from the patients. In this study none of the patients in control group were free of *H. pylori* in the stomach 11 months after completion of therapy. Goddard and Logan (1995) observed OCM - omeprazole, clarithromycin, nitroimidazole - triple therapy as the most effective, single, well-tolerated anti-*H. pylori* treatment.

Vakil and Go (2000) reviewed various treatment strategies developed for *H. pylori* in different clinical outcomes and paediatric population. de Boer et al. (2000) achieved 94.4% cure by administering a single capsule containing 60 mg colloidal bismuth subcitrate, 125 mg tetracycline, and 125 mg metronidazole. Hayashi et al. (2000) reported the synergistic anti-adhesion activity of rabamipide and ecabet sodium (the anti-ulcer agents) against *H. pylori*.

Lachman and Howden (2000) reported that subject's *H. pylori* status did not affect the antisecretory effect of ranitidine. Breiter et al. (2000) showed that rabeprazole 20 mg q.a.m was superior to ranitidine 150 mg b.i.d in healing, resolving ulcer pain frequency, improving night time pain severity, and improving overall well being. Pantoprazole was shown to be more effective than ranitidine in the treatment of DU and it provided faster ulcer healing in 97.1% of patients in 4 weeks.

The effectiveness of medicinal plants for eradication of *H. pylori* was evaluated. An alkyl methyl quinolone (AM quinolone) alkaloid from Gosyuyu, a Chinese herbal medicine, was found to be useful, and the MIC of these alkaloids against *H. pylori* was reported to be similar to the MIC of amoxycillin and clarithromycin (Hamasaki, et al., 2000). Some tea and

rosemary extracts were found to clearly inhibit *H. pylori* urease *in vitro* and green tea extract showed strongest inhibition of *H. pylori* urease among the 77 plant derived foodstuff samples tested (Matsubara et al., 2003). In a dose-dependent manner the *H. pylori* gastritis and prevalence of infection were suppressed in green tea extract fed Mongolian gerbils. So it was thought that the eradication of *H. pylori* could be achieved without affecting other intestinal flora by using such plant products.

It was found that 24-31 h were sufficient incubation time for routine *in vitro* resistance test for *H. pylori* when conditions were optimal - i.e., microaerophilic atmosphere, a good medium, bacteria that are harvested during an active phase of growth, and staff trained in this procedure (Henriksen et al., 1997).

Although the ideal therapy has not yet been identified, many factors that may influence outcome have been identified, making the design of definitive studies possible (Graham. 2000). In a recent study, Malaty et al. (2002) observed that most newly acquired *H. pylori* infections happened before age 10 years. So treatment and preventive strategies should be aimed at children in this age group.

Even though the isolation of *H. pylori* was succeeded more than 20 years ago, there are no reports from Kerala regarding the prevalence or isolation of the organism. This prompted us to focus on the standardization of the method of isolation of *H. pylori* from human gastric biopsy samples.

Several studies proved the association of *H. pylori* infection with gastric cancer. The scientific community continues to unravel the mechanism by which this chronic infection leads to gastric cancer. The seroprevalence studies, especially the prevalence of anti-*H. pylori* IgG , were mainly used to elucidate the association of *H. pylori* infection with various gastroduodenal pathologies. Several major antigenic structures of the organism have already identified. But the inductions of IgG subclasses against *H. pylori* antigens

were not studied in detail either *in vitro* or *in vivo*. In this circumstance, we have tried to analyze the induction of IgG subclasses against different antigens prepared from clinical isolates of *H. pylori in vitro* conditions. We also analyzed the level of anti-*H. pylori* IgG and IgG subclasses in gastric cancer patients serum samples.

Enhanced neutrophil/macrophage activities were proposed to be a critical factor in the pathogenesis of *H. pylori* infections. Moreover, the molecular pathways leading to the gastric carcinogenesis clearly shows the involvement of ROS derived from activated cells. In this context, the activation of murine macrophages with the antigens from the clinical isolates of *H. pylori* was also studied.

Recent reports showed an increased lipid profile in *H. pylori* positive subjects and correlation of *H. pylori* infection with coronary heart disease was observed. In contrast with this hypocholesterolemia was observed in cancer patients. This prompted us to analyze the lipid profile of gastric cancer patients with respect to *H. pylori* infection.

Keeping the above information in mind, it was of great interest to investigate the following objectives:

1. To isolate *H. pylori* from human gastric biopsy samples.
2. Prepare the *H. pylori* antigens and examining - (a) the effect of these antigens on *in vitro* proliferation of human mononuclear cells and (b) the effect of *H. pylori* antigens in the induction of human IgG subclasses *in vitro*.
3. Analyzing the levels of immunoglobulin G subclasses in anti-*H. pylori* IgG positive and negative gastric cancer patients.
4. Effect of *H. pylori* antigens in murine macrophage activation, and
5. To analyze the lipid profile in sera of anti-*H. pylori* IgG positive and negative gastric cancer patients.

Chapter II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

ISOLATION OF *HELICOBACTER PYLORI* FROM GASTRIC BIOPSY SAMPLES

Collection of biopsy samples

The patients, who were pathologically (endoscopy) diagnosed for gastric cancer and peptic ulcer, were chosen for the collection of biopsy samples. These patients were the out patients of Baby Memorial Hospital, Calicut.

Transportation of specimen

Immediately after collection, the biopsies were transferred to sterile thioglycollate broth containing casein enzymatic hydrolysate (1.5%), dextrose (0.55%). sodium chloride (0.25%), L-cystein (0.05%), sodium thioglycollate (0.05%), Nalidixic acid (0.005%), and vancomycin (0.001%). The transportation to the laboratory was carried out at 4°C in a mini-cooler.

Processing of biopsy specimens

Rapid urease test (RUT)

All processing and cultures were performed in a vertical air laminar flow (Kirloskar Electrodyne Ltd.)

The biopsy specimens were divided into two halves. One part was used for RUT and the other part for isolation of *H. pylori*. The method of McNulty et al. (1989) was followed for RUT. One part of the biopsy from each patient was placed in a test tube with 1 ml. of sterile rapid urease test media containing 2% urea, 0.2% dipotassium hydrogen phosphate, 0.5% sodium chloride and 0.004% phenol red (pH 6.8). The tubes were incubated at room temperature up to 24 h and the results were characterised on the basis of the colour change of the medium. The biopsies were considered as strongly positive, moderately positive and weakly positive if the positive result

developed within 6, 12 and 24 h of incubation respectively. The results were noted as negative if there is no colour change produced even at 24 h of incubation.

Isolation of *H. pylori*

The second part of the biopsy sample of each patient was processed for isolation of *H. pylori* in culture. By using sterile forceps, the specimen was placed on a sterile microscopic slide and 2-3 drops of thioglycollate broth was added. The tissue was teased into fine spreads with forceps and needle. This was then streaked on chocolate agar plates containing 0.005% nalidixic acid, 0.001% vancomycin, and 0.005% trimethoprim. The plates were incubated either in a CO_2 incubator (ShelLab Model TC 2323) or in an anaerobic jar using gas-generating pack (Anaerocult C from EMERCK, Germany). The incubation was done at 37°C in an atmosphere containing 10% CO_2 and 95% humidity for 4 days. The unused tissue macerate on the slide was subjected to gram's staining. The photograph of the biopsy specimen showing the organism was taken using Leitz Dialux 20 Microscope with photographic facility.

Characterization of Isolates

Characteristics of each colony formed on the culture plates were noted and subjected to Gram's staining. Those colonies, which showed characteristic cell morphology of *H. pylori.* were selected and subculture made on fresh plates. The pure cultures of each isolate were used for the following biochemical characterization.

a) Catalase test: The method of Taylor and Achanzar (1972) was adopted. A small portion of the colony was picked with a sterile capillary tube and immersed in H_2O_2 solution (10% v/v) in a tube. The generation of effervescence noted as positive reaction.

b) Oxidase test: The method reported by Steel (1961) was adopted. The isolate was taken with the sterile capillary tube and rubbed on a filter paper impregnated with 1% solution of NNNN'-tetra methyl para phenylene diamine dihydrochloride. The development of purple colour within 10 s is considered as positive reaction.

c) Urease test: The test was performed on Christensen's urease medium (Christensen, 1946). The media was prepared and the colonies were inoculated on to the slants. Incubation was carried out in a CO_2 incubator with 10% CO_2 at 37°C. The colour change of the media from golden yellow to pink was noted as positive reaction.

The cultures were also tested for its ability to utilize glucose and nitrate reduction. Motility of the organism was tested.

The biochemical characters expressed by the colonies were compared with the characteristics of *H. pylori* reported in standard references. The characters were also compared with those of a standard strain (HP 101) obtained from Dr. A. Grob (Germany).

ANALYSIS OF ANTI-*H. PYLORI* IgG AND IgG SUBCLASSES IN GASTRIC CANCER PATIENT SERA

Collection of serum samples

Serum from gastric cancer patients, who were the inpatients of Regional Cancer Centre, Thiruvananthapuram or Medical College Hospital, Calicut, was collected for the analysis of anti-*H. pylori* IgG and IgG subclasses. Only those cases, which were diagnosed as gastric cancer, were selected. Patients at different age group from 24-75 were included in the present study. Age matched healthy persons were included as controls.

5 ml Blood from each patient was taken by venipuncture and allowed to clot and serum was separated by centrifuging at 900 g for 10 min in a tabletop centrifuge. The separated serum was stored at $^{-}$20°C in a freezer until used for the assay.

Anti-*H. pylori* IgG Analysis

The level of anti-*H. pylori* IgG in the serum samples were analysed by using commercially available diagnostic kits (UBI Magiwel EIA, United Biotech Inc., Mountain

view, CA 94041-1517) which has the sensitivity and specificity of 97% and 93% respectively.

The assay was done as described below. 100 µl of serum samples at 1:101 dilutions was added to pre-coated wells with *H. pylori* antigens. Calibrator, positive control and blank were maintained in the assay. The plates incubated at room temperature for 30 min. After the incubation, the wells were washed five times with wash buffer. 100 µl of enzyme conjugate (anti-human IgG-HRP conjugate) was added to each well and the plates were incubated for 30 min. After washing the plate, 100 µl of substrate with chromogen was added to the wells. The colour was developed by incubating the plates for 15 min at room temperature. 50 µl of 1 N H_2SO_4 was added to the wells to stop the reaction and the plates were read in an ELISA reader at 450nm.

The amount of anti-*H. pylori* IgG in each patient sample was calculated by using the formula given by the manufacturer

$$\text{Anti-}H.\ pylori\ \text{IgG (EU/ml sample)} = \frac{\text{O.D. of the test sample}}{\text{O.D. of the calibrator}} \text{ X EU/ml of calibrator}$$

The samples were considered positive for *H. pylori* antibodies if the sample contains 40 or above EU/ml.

Analysis of IgG subclasses in serum samples

Purified monoclonal antibodies specific to human IgG subclasses, such as HG-11 for IgG_1 (Mayus et al., 1986), Hp-6016 for IgG_2, Hp-6066 for IgG_3, and Hp-6011 for IgG_4 were used for coating the micro titre plate. Specific myeloma proteins W-6, W-238, W-242 and W-210 for IgG_1, IgG_2, IgG_3 and IgG_4 respectively were used as subclass specific standards and WHO 67/97, a pooled serum assigned for IgG subclasses, was used as reference serum sample. All the above reagents were a gift from Dr. M. H. Nahm, Washington University, School of Medicine, St. Louis, Missouri, U.S.A. Goat anti-human IgG peroxidase conjugate was a gift from Immunological Lab. National Institute of Immunology, New Delhi.

Micro titre plates were purchased from Laxbro, product of Bhagawandas and Company at Pune. Bovine serum albumin (BSA), and o-phenylene diamine dihydrochloride (OPD) were purchased from Sigma chemicals, St. Louis, U. S. A. All other reagents used in the assay were purchased from MERCK/Qualigens, India.

Optimization of ELISA System

The method of Butch et al. (1989) was adopted as the basis for optimisation of ELISA system. 5, 10, and 20 µg/ml concentrations of subclass specific monoclonal antibodies were prepared in 0.1 M carbonate buffer (pH 9.6) and 150 µl of each concentration was used for coating micro titre plates. The experiments were done in quadruplicates. The plates were incubated at room temperature for 3 h followed by washing with 0.05% Tween-20 in 10 mM PBS (pH 7.2). The wells were blocked with 200 µl of 1% BSA in PBS with 0.05% Tween-20 for 30 min. The plates were washed and 150 µl of appropriately diluted serum samples, pooled normal serum (n=50) and WHO-67/97 reference serum (as quality control) were added into different wells. 150 µl of subclass specific myeloma proteins ranging from 2–100 ng concentrations were used as standards. The plates were incubated at room temperature for 3 h. Washing step was repeated. 150 µl of different dilutions of goat-antihuman HRP conjugate (1:2500, 1:5000, 1:10000) were added to the wells as checkerboard and incubated further 3 h at room temperature. Washed the plates and 150 µl of the substrate (0.04% OPD in 0.1 M citrate buffer, pH 5 mixed with H_2O_2) was added and kept in dark at room temperature for 20 min. The reaction was stopped by addition of 75 µl of 1N H_2SO_4 to each well.

The plates were read in an ELISA reader (Biotech Model EL_X 800, Biotech Instruments Inc.) On the basis of data obtained it was decided to choose 10 µg/ml subclass specific monoclonal antibodies and 1:5000 dilution of secondary antibody conjugates for the analysis of IgG subclasses.

ELISA for IgG Subclasses

150 µls of 10 µg/ml solutions of HG-11, Hp 6016, Hp 6066, and Hp 6011 were used to coat the micro titre plate for IgG_1, IgG_2, IgG_3 and IgG_4 respectively. Serum samples were diluted to 1:40000 for IgG_1, 1:20000 for IgG_2, 1:5000 for IgG_3 and 1:100 for IgG_4 with 1% BSA in 10 mM PBS (pH 7.2). 225 µl of each dilution of the serum sample were added to the first wells of the plates for IgG_1, IgG_2, IgG_3 and IgG_4 assay. It was then serially diluted 3-fold in the subsequent wells up to 6 wells i.e., 75 µl from the first well was taken and transferred to the next well containing 150 µl of BSA solution with the help of a micropipette (Glaxo, Germany). 150 µl of 1:5000 dilution of secondary antibody enzyme conjugate was added to each well. All other steps were same as described in optimisation of ELISA. In each plate pooled serum samples, standards for respective subclasses and WHO-67/97 reference serum were included as quality controls.

The concentration of each subclass in the sample was determined from standard graph plotted in the semi-log paper by comparison to the optical density generated by standard proteins. The sensitivity of the assay was 2 ng/ml.

PREPARATION OF ANTIGENS FROM *H. pylori* STRAINS (ISOLATES)

Different isolates of *H. pylori* were grown on chocolate agar containing antibiotic supplement. After incubation for 48 h in microaerophilic conditions, the cells were harvested by scraping and were washed twice with PBS (0.1 M, pH 7.2) by centrifugation at 8000 rpm for 15 min at 4°C in a refrigerated centrifuge (Plastocraft Superspin-R model) and various methods used for the preparation of antigens.

Acid-Glycine Extraction (AG)

The method described by Hirschl et al. (1988) was followed with some modifications. 2.5 ml of 0.2 M glycine-HCl buffer (pH 2.2) was added to 100 mg (wet weight) *H. pylori* cells. The suspension was mixed gently at room temperature for 20 min. It was then centrifuged at 15000 rpm for 15 min at 4°C in a refrigerated centrifuge.

The supernatant was neutralized by adding 1 M NaOH and dialyzed against PBS for 24 h at 4°C. Particles, if any, were centrifuged out and the preparation was lyophilised in a Savant lyophilizer (RVT 400 model) and stored at -80°C deep freezer (NUAIRE, NU2500E)

n-Octyl-β-D-Glucopyranoside Extraction (NOG)

The method of Evans Jr. et al. (1989) was adopted for n-octyl-β-D-glucopyranoside extraction of antigens from *H. pylori*. The cell suspension was centrifuged at 8000 rpm for 15 min at 4°C. The pellet was suspended in 2.5 ml of 1% solution of n-octyl-β-D glucopyranoside (Sigma Chemicals, St. Louis, USA) in sterile PBS (pH 7.2). After incubation for 20 min at room temperature, the suspension was centrifuged at 12000 rpm for 15 min at 4°C. The extract was dialysed against half concentrated PBS (0.05 M, pH 7.2) at 4°C followed by dialysis against distilled water at 4°C. The particles, if any, were centrifuged out and the supernatant lyophilised and stored at -80°C in a deep freezer.

Whole Cell Sonicate Preparation (WCS)

Method described by Hirschl et al. (1988) was followed. The cell suspension was washed twice in PBS and it was inactivated at 60°C for 15 min. The inactivated cells were resuspended in PBS and sonicated 4 x 10 seconds by keeping in an icebox. The sonicate was centrifuged at 12000 rpm for 20 min and the supernatant concentrated by using a lyophilizer (Savant, RVT 400 model). The preparation was stored at -80°C in a deep freezer till used for further experiments.

Hot Phenol-Water Extraction (LPS)

The method of Westphal and Khan (1965) was adopted for hot phenol-water extraction. The cells from 25 plates were harvested into PBS and washed as described earlier. The pellet was resuspended in water and an equal amount of 90% phenol (pre-heated to 65-68°C) was added. The mixture was vigorously shaken and kept at 65°C. After incubation for 15 min it was cooled to 10°C and centrifuged at 12000 rpm

for 20 min. The water layer was collected and the extraction procedure was repeated twice. The water-extracted layers were pooled and dialysed against distilled water to remove any residual phenol (48 h at room temperature with four changes of water). Suspended particles, if any, were removed by centrifugation at 12000 rpm for 10 min. The supernatant lyophilised and stored at $^{-}$80°C in a deep freezer.

Sodiumdodecyl sulphate-Polyacrylamide gel Electrophoresis (SDS-PAGE)

Protein profile of each antigen preparations, except hot phenol-water extract, was determined by SDS-PAGE. The method of Laemli (1970) was adopted. The separation was carried out in a 10.5% separating gel with 5% stacking gel. For preparing separating gel 3.03 ml double distilled water, 2.25 ml 1.5 M Tris (pH 8.8), 0.9 ml 10% SDS, 0.46 ml glycerol and 3.15 ml 30% Acrylamide-0.8% bis-acrylamide were mixed. Before casting the gel 6 µl of TEMED, and 60 µl of 10% ammonium per sulphate solutions were added to the mixture.

After polymerisation, the gel was washed thrice with distilled water. The stacking gel was prepared by mixing 1 ml 0.5 M Tris (pH 6.8), 40 µl of 10% SDS, 667 µl of 30% acrylamide-0.8% bis-acrylamide, 2.24 ml double distilled water, 4 µl TEMED, and 53 µl of 10% ammonium per sulphate. A comb was placed and the stacking gel was prepared. 100 µl of each sample (containing 100 µg protein) was mixed with 50 µl of sample buffer containing SDS, glycerol, EDTA, 0.5 M Tris (pH 6.8) and bromophenol blue in double distilled water. The tubes were kept in boiling water bath for 3 min. The wells of the gels washed thrice with running buffer (Tris-glycine, pH 8.3) and 25 µl of the sample was added to each well. Molecular weight markers (Medium range, purchased from Bangalore Genei Pvt. Ltd.) were also included in the experimental protocol. The electrophoresis was carried out in BioRad Mini Gel Unit (BioRad, Hong Kong) at a constant voltage (100 V) using Hoeffer power supply (Hoeffer Scientific Instruments, Sanfrancisco, California). After the electrophoresis the gels were stained

with Coomassie Brilliant Blue- R250 (BioRad) followed by destaining with a mixture of methanol, acetic acid, and water.

IN VITRO SECRETION OF IgG SUBCLASSES

Isolation of mononuclear cells from tonsils

Fresh human tonsils were chosen for obtaining mononuclear cells for the present study because it will contain 60-70% B-cells, 30-40% T-cells, 1-8% monocytes and <1% dendritic cells. Thus a combination of all cells required for adequate humoral response will be available for in vitro studies.

Immediately after tonsillectomy, the specimens were collected and transported to the laboratory at 4°C. The processing of the specimens was done within 2 h of collection.

Processing of the tonsils

Protocol described by Coligan et al. (1996) was followed. Briefly, the specimens were placed on a sterile stainless steel sieve, which was set on a sterile petridish. The tissue was cut into 3-5 mm pieces and squeezed through the mesh using flat end of a syringe plunger.

The cell suspension was transferred to 50 ml sterile centrifuge tubes and allowed to stand 5 min. The debris was removed. The suspension over-layered on Ficol-Hypaque gradient (density 1.007 g/L) (Pharmacia Biotech, Upsala, Sweden) and centrifuged at 900 g for 20 min at room temperature. The layer of mononuclear cells (Buffy coat) at the interface was collected and transferred to fresh sterile centrifuge tube. The cells were washed three times with sterile PBS. The final washing was done with RPMI medium.

The cell pellet was re-suspended in complete RPMI medium and checked the viability with trypan blue as described by Bortran and Vetvika (1995) and counted the number of cells with a haemocytometer. The cell density was adjusted to 2×10^6 cells/ml.

Proliferation assay

200 µl of the above cell suspension was dispensed into each well of a 48 well culture plate (Costar, Sigma, St. Luis, USA). Con A (purchased from CSIR Centre for Biochemicals, New Delhi) and PHA (purchased from HiMedia laboratories, Mumbai) were used as mitogens. Different concentrations of mitogens, such as 10, 20, 30, 40, 50, 60, 70, and 80 µg/ml. were added to the wells. Similarly, different concentrations of *H. pylori* antigens obtained from various preparations were also added to each well. Control wells were also included in the protocol without mitogens or antigens. The plates were incubated at 37°C at 5% CO_2 atmosphere and 95% humidity (NUAIRE, IR auto flow-water jacketed) for 72 h.

The proliferation of cells in cultures was assayed by using 3-(4,5-dimethyl-2-thioazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Mosman, 1983). At the end of incubation, 20 µl of sterile MTT solution (5 mg/ml in PBS) was added to each well and incubated for 4 h at 37°C followed by the addition of 200 µl of acidic isopropanol (0.04 N HCl in Isopropanol) to each well and kept at room temperature to dissolve all crystals formed (5-10 min). After mixing the content of the wells, optical density was measured at 570 nm in a Spectrophotometer (Shimadsu, UV 1601 model) with 0.5 ml cuvettes.

Assay of IgG subclasses

The cultures were prepared as described above. After incubation for 7 days, the level of IgG subclasses in the culture soup was assayed by following ELISA technique (Butch et al., 1989). The protocol was same as that described in the assay of IgG subclasses in serum samples, except for the sample dilution. The culture soup was diluted 2 times for estimating the IgG subclasses. The level of each subclass was determined from the standard graph generated for each subclass.

MACROPHAGE ACTIVATION STUDIES

Collection of murine peritoneal exudates cells (PEC)

Peritoneal cavity of mice provides an accessible site for the harvest of resident macrophages. Generally the normal mouse peritoneal cavity will yield mature, resident macrophages.

The protocol described by Coligan et al., 1996) was adopted. The mice were killed by cervical dislocation. Making an incision followed by peeling off the skin was performed to expose the abdominal wall. 5 ml complete RPMI medium containing 5 µg/ml heparin was injected into the peritoneal cavity and the injected medium was circulated into the cavity by gentle massage. The medium was aspirated with the help of a syringe and collected into 15 ml centrifuge tubes. The suspension was centrifuged at 4000 rpm for 15 min and the pellet was washed with RPMI medium and re-suspended in complete RPMI medium. The viability of the cells was checked with trypan blue. The cell numbers were counted by using a haemocytometer and the cell density was adjusted to 1 x 10^6 cells/ml.

100 µl of PEC suspension was dispensed in each well of a 96 well culture plate (TARSONS Products Pvt. Ltd. Kolkata) and placed in a humid CO_2 incubator for 3 h at 37°C. The medium was removed and the adherent cells were washed with RPMI medium. The wells without cell suspension and only with CRPMI medium served as controls. 100 µl of different concentrations of antigens (5, 10, 20, 30, 40, and 50 µg/ml) and mitogens (5, 10, 20, 30, 40, and 50 µg/ml) in complete RPMI medium were added into respective wells. Each concentration was prepared in triplicates. The plates were incubated at 37°C in an incubator with 5% CO_2 and 95% humidity.

Assay of nitric oxide (NO)

The method described by Coligan et al., 1996) was adopted. 200 µl culture supernatant was mixed with 200 µl Griess reagent (an equal mixture of

1% sulphanilamide in 5% orthophosphoric acid and 0.1% naphthyl ethylene diamine in distilled water) in separate tubes and incubated at room temperature for 20 min.

Standard was prepared by taking sodium nitrite at the range of 50-500 ng in CRPMI medium and treated similar to that of test samples. The blank was included in the experimental protocol. The optical density of the colour developed was measured at 540 nm in a spectrophotometer with 0.5 ml cuvettes.

Standard graph was plotted and the concentration of NO^{2-} in the sample was read from the graph. The amount of NO formed was expressed in $nM/10^6$ cells/ 24 h.

Release of enzymes from Macrophages

After removing culture soup from each well, the adherent macrophages were washed three times with sterile normal saline. 50 µl of cold double distilled water was dispensed into each wells and kept at 4°C overnight. The cell lysate was taken in the eppendorff tubes and centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was used for the enzyme assay.

Assay of Acid Phosphatase

The method described by Burch (1969) was adopted with following modifications. Briefly, 100 µl of acetate buffer (0.1 M, pH 5) and 30 µl of double distilled water were dispensed into each well of a micro titre plate. 40 µl of the enzyme preparation from the macrophages was added to the wells. Mixed well and 25 µl substrate (p-Nitro phenyl phosphate - 0.05 M) was added to all wells, except control wells. The plates were incubated for 1 h at 37°C in a humid chamber. After incubation, the reaction was stopped by adding 40 µl of 6 N NaOH to each well, including control wells. 25 µl substrate was added to the control wells. The experiments were conducted in duplicates. The intensity of colour developed was measured at 420 nm in a Spectrophotometer with 0.5 ml cuvettes.

A standard graph was prepared by using different concentrations of p-Nitrophenol as standard. The level of enzyme activity in presence of different antigen

preparations and mitogens were read from the standard graph and expressed as nM of p-Nitrophenol formed/10^6 cells/ h.

Assay of β-Glucouronidase

The method described by Fishman and Bernfeld (1968) was adopted with the following modifications. Briefly, 25 µl acetate buffer (0.1 M, pH 5) was dispensed into each wells of a micro titre plate. 50 µl of enzyme preparation from macrophages was added to the wells in duplicates. 25 µl substrate (Phenolphthalein glucouronic acid - 1 mg/ml) was added to each well, except in control wells. The plate was incubated at 37°C in a humid chamber for 1 h. The reactions was stopped by adding 100 µl of stopmixture, which contain 1.503 g glycine, 5.299 g sodium carbonate, and 0.6 g sodium hydroxide in 100 ml distilled water, including in control wells. 25 µl of substrate was added to control wells. The intensity of the colour developed was measured at 540 nm in a spectrophotometer with 0.5 ml cuvettes.

A standard graph was prepared by using different concentrations of phenolphthalein as standard. The enzyme activity in the presence of antigen preparations and mitogens were read from the standard graph and expressed as nM phenolphthalein formed/10^6 cells/h.

ANALYSIS OF LIPID PROFILE

Estimation of total cholesterol in serum samples (Cholesterol oxidase-Peroxidase method)

Commercially available diagnostic kits purchased from Span diagnostics, Surat, India were used to assess the total cholesterol present in the serum sample of patients. 10 µl of serum sample and 10 µl of standard were added to 1 ml of cholesterol reagent in separate tubes. All tubes were mixed well and incubated at 37°C for 10 min. The optical density of the colour developed by samples and standards were measured against reagent blank, which contain only cholesterol reagent, at 505 nm using a UV-Visible spectrophotometer (Shimadsu, UV1601 model).

The method is based on the following enzymatic reactions:

$$\text{Cholesterol esters} \xrightarrow{\textit{Cholesterolesterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\textit{Cholesteroloxidase}} \text{Cholesten-3-one} + H_2O_2$$

$$H_2O_2 + \text{4-AAP} \xrightarrow{\textit{Peroxidase}} \text{Quinoneimine} + H_2O$$

The amount of total cholesterol in the samples was calculated by using the following formula.

$$\text{Total cholesterol (mg/dL)} = \frac{\text{Absorbance of the test x 200}}{\text{Absorbance of the standard}}$$

Estimation of HDL-cholesterol

Commercially available diagnostic kits purchased from Span diagnostics, Surat, India were used for the estimation of HDL-cholesterol. 0.3 ml of the precipitating reagent (Polyethylene glycol 6000) was mixed with 0.3 ml of the sample. After incubating for 10 min in room temperature, the tubes were centrifuged at 4000 rpm for 10 min and the clear supernatant was collected. 1ml of cholesterol reagent was added to 100 µl of the supernatant and 100 µl of the HDL standard and incubated at 37°C for 10 min. The optical density of the colour developed was measured at 505 nm in a spectrophotometer against the reagent blank, which contain only cholesterol reagent.

The level of HDL cholesterol was calculated by using the following formula.

$$\text{HDL Cholesterol (mg/dL)} = \frac{\text{Absorbance of the test x 100}}{\text{Absorbance of standard}}$$

Estimation of triglycerides

Commercially available diagnostic kits purchased from Span diagnostics, Surat, India was used for the estimation of triglycerides in patient sera. Manufacturer's instructions were followed to perform the test. 10 µl of sample and 10 µl of triglyceride standard were added to 1 ml of triglyceride reagent in separate tubes. Incubated at 37°C for 10 min. The optical density of the colour developed was measured at 505 nm

in spectrophotometer against the reagent blank, which contain 1 ml triglyceride reagent only.

The method of estimation is based on the following enzymatic reactions

$$\text{Triglycerides} \xrightarrow{\textit{Lipoproteinlipase}} \text{Glycerol + Free fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\textit{Glycerolkinase}} \text{Glycerol-3-phosphate + ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\textit{Glycerol-3-phosphatae}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4- AAP} \xrightarrow{\textit{Peroxidase}} \text{Quinoneimine} + H_2O$$

The amount of triglycerides in the samples was calculated by using the formula.

$$\text{Total triglycerides (mg/dL)} = \left[\frac{\text{Absorbance of the test X 200}}{\text{Absorbance of the standard}}\right]$$

Estimation of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL)

The formula derived by Friedewald et al. (1972) was used to calculate cholesterol in VLDL and LDL fractions in serum samples.

$$\text{Cholesterol in VLDL fraction} = \frac{\text{Total triglycerides}}{5}$$

$$\text{Cholesterol in LDL fraction} = \text{Total cholesterol} - \left[\frac{\text{Total tiglycerides}}{5} + \text{HDL}\right]$$

Chapter III

ISOLATION OF *HELICOBACTER PYLORI* FROM GASTRIC BIOPSY SPECIMENS

CHAPTER III

ISOLATION OF *HELICOBACTER PYLORI* FROM GASTRIC BIOPSY SPECIMENS

Gastric pH of healthy humans is below 2 and this makes the colonization of microbes within the stomach difficult. Except for the presence of microbes originating from food, the human gastric content is sterile. This led to the thought that stomach is free from infectious diseases. But the successful isolation of *H. pylori* from gastric biopsies by Marshall and Warren (1984), and the accumulated report on its association with various gastroduodenal pathologies abolished this concept.

The key factor in the success of cultivation of *H. pylori* from gastric biopsy was the establishment of microaerophilic conditions originating from the technique applied for *Campylobacters*. Marshall and Warren (1984) inoculated the minced gastric tissue on non-selective blood agar and chocolate agar and incubated at 37°C under microaerophilic conditions. The colonies were developed on the plates after four days of incubation. These colonies were identified as *H. pylori*.

In tissue sections *H. pylori* appears as unipolar, multiflagellate, rod-like organism with blunty rounded ends. They measure 0.5-1.0 µm in width and 2.5-4.0 µm in length (Goodwin et al., 1985; Jones et al., 1985). Direct observation on colonizing human gastric mucosa, it is a curved, sinuous or gently spiral bacterium (Goodwin and Armstrong, 1990). The sheathed flagella that terminate in a bulbous disc structure are the most distinguishing structural characteristic of the organism (Goodwin et al., 1985).

In cultures, *H. pylori* occur usually in the form of straight or slightly curved rods. Prolonged culture give rise to emergence of coccoid forms. Such forms occur in the faeces of the infected subjects. These are usually non-culturable forms of *H. pylori* (Bode et al., 1993). Organisms in the stationary phase of culture of *H. pylori* changed

their morphology from the spiral to coccoid form markedly under the anaerobic conditions (Shirai et al., 2000). Cellini et al. (1994) demonstrated the viability of the coccoid form by using BALB/c mice model. The conversion of coccoid form to spiral form and the recovery of urease activity and the ability to be subcultured on solid media were reported by Anderson et al. (1997). Thus, the coccoid forms of *H. pylori* were thought to be dormant form of the bacterium, which can revert to spiral form when conditions are appropriate.

The growth of *H. pylori* was improved by the incorporation of blood in the culture media (Caudron and Kirby, 1989). For the primary isolation of the organism from clinical specimens, blood based solid media were used. Marshall and Warren (1984) used chocolate agar for the isolation of the organism. Dunn et al. (1991), Doig et al. (1992), Lin et al. (1992), Pronovost et al. (1994), Cellini et al. (1994) and several others used chocolate agar for the cultivation of *H. pylori*. Skirrow's blood agar (Bolin et al., 1995; Nishiya et al., 1999), Blood agar (Kawanishi et al., 1995; Evans Jr. et al., 1989. Pan et al., 1997; Xiang et al., 2000), Brain heart infusion agar (Young et al., 2000; Rasko et al., 2000), Mueller-Hinton agar (Kostrzynska et al., 1991) were also used for primary isolation. Nakao et al. (1997) used Trypticase soy agar containing 5% sheep blood and Taylor (1988) used Brucella agar containing 10% sheep blood for culturing *H. pylori*. An indicator medium, Belo-Horizonte Medium (BHM), was developed (Queiroz et al., 1987) by incorporating triphenyl tetrazolium chloride to brain-heart infusion sheep blood agar. On this medium *H. pylori* developed colonies with unique golden pigmentation.

Some authors used blood free media for the cultivation of *H. pylori*. Morshed et al. (1994) obtained good growth of the organism in Brucella broth supplemented with 1% heated horse serum and 0.1% β-cyclodextrin. Egg yolk emulsion agar is another blood free medium reported (Westblom et al., 1991).

Testerman et al. (2001) reported chemically defined medium (F-12 agar), which supported the growth of *H. pylori*. F-12 agar plus cholesterol or β-cyclodextrin represents the first transparent chemically defined medium and the first urease indicator agar for *H. pylori*.

H. pylori grow slowly in broth media (Westblom et al., 1991). Contaminating microorganisms usually grow much faster than *H. pylori* and make liquid media useless for primary isolation of *H. pylori* from gastric biopsies.

In the absence of inflammation *Helicobacter* species appeared to be a part of a complex, indigenous microbial flora found in the gastric biopsy specimen (Monstein et al., 2000). Several authors used antibiotic supplement in the media for inhibiting the growth of contaminants. *H. pylori* are naturally resistant to vancomycin, cefsulodin, trimethoprim and sulfonamides (Goodwin and Armstrong, 1990). Commonly vancomycin, trimethoprim and nalidixic acid were used as antibiotic supplements in the media for the selective isolation of *H. pylori*. Resistance to nalidixic acid has been reported as a feature of *H. pylori*, but a few strains are sensitive. Taylor (1988) observed that most strains grown on 40 mg/L of nalidixic acid, but all are inhibited at 48 mg/L.

Primary cultures of *H. pylori* have less oxygen tolerance than most *Campylobacter* species, with a growth maximum at 3-7% of oxygen (Andersen and Wadstrom, 2001). Usually *H. pylori* were grown in jars with gas generating kits (Marshall and Warren, 1984; Anderson et al., 1997; Young et al., 2000) or in a CO_2 incubator with a standard microaerobic atmosphere (Kostrzynska et al., 1991; Testerman et al., 2001).

Most of the authors used gastric biopsies for the isolation of *H. pylori*. But there are reports showing the presence of *H. pylori* in oral cavity and faeces. Krajden et al. (1989) and Cellini et al. (1995) reported the isolation of *H. pylori* from dental plaques. They also reported that the isolates were identical with that colonized in the stomach of

respective patients. Ferguson et al. (1993) isolated *H pylori* from saliva. *H. pylori* were cultured from gastric juice of the patients (Young et al., 2000). Some authors isolated *H. pylori* from faeces also (Thomas et al., 1992). But in all these cases the rate of isolation was very poor. Therefore, the major specimen that is used for the isolation of *H. pylori* is gastric biopsies.

For inoculation, the biopsy sample can be rubbed several times on solid media (Jones et al, 1984), but mincing or grinding in the transport medium with a glass grinder results in heavier, more uniform growth (Goodwin et al., 1985).

RESULTS

Biopsy samples were collected from 41 patients with different gastroduodenal symptoms as per the endoscopy findings (Table 3.1). These patients were at the age range of 15-80. Of the 41 samples, majority was of duodenal ulcers. Two samples were from normal gastric mucosa.

Table 3.1 Data on biopsy specimens

Endoscopic findings	No. of cases	RUT positive	Culture positive
Duodenal ulcer	13	08	04
Healing duodenal ulcer	16	07	04
Duodenitis	06	05	03
Gastritis	02	02	01
Mucosa oedematous	01	Nil	Nil
Fundal carcinoma	01	Nil	Nil
Normal	02	Nil	Nil

Overall, 53.65% cases (22/41) showed positive reaction in rapid urease test (Table 3.2). The positivity of the sample was defined as follows: those biopsy

specimens, which gave positive result within 6, 12 and 24 h were recorded as strong positive, moderately positive and weakly positive, respectively. Both biopsies from patients with normal mucosa were negative for rapid urease test and both gastritis biopsies were positive. 83.33% of duodenitis cases (5/6), 61.53% of duodenal ulcer cases (8/13), and 43.75% healing duodenal ulcer cases (7/16) were positive for rapid urease test.

Table 3.2 Rapid urease tests in Biopsies

Total No. tested	**41**
Strong positive	15
Moderately positive	05
Slightly positive	02
Negative	19

The colonies developed on the agar were analyzed and those colonies, which showed characteristic morphology and strong positive reactions for catalase, oxidase and urease tests were identified as *H. pylori* (Table 3.3). Further characterization of these colonies showed that they were unable to grow aerobically and does not utilized glucose or other carbohydrates. All isolates showed active cork-screw type motility.

Urease negative biopsies did not yield any *H. pylori* colonies. Total of 12 strains were isolated from rapid urease test positive biopsy samples.

Figure 3.1 shows the presence of *H. pylori* in the Gram-stained preparations of biopsy specimens used for the isolation of the organism. Typical morphology like slightly curved structure of *H. pylori* can be seen in the smear. Figures 3.2 and 3.3 shows the development of *H. pylori* colonies on chocolate agar and Gram-stained smear of isolated *H. pylori* colonies, respectively. Gram's stained smear from isolated

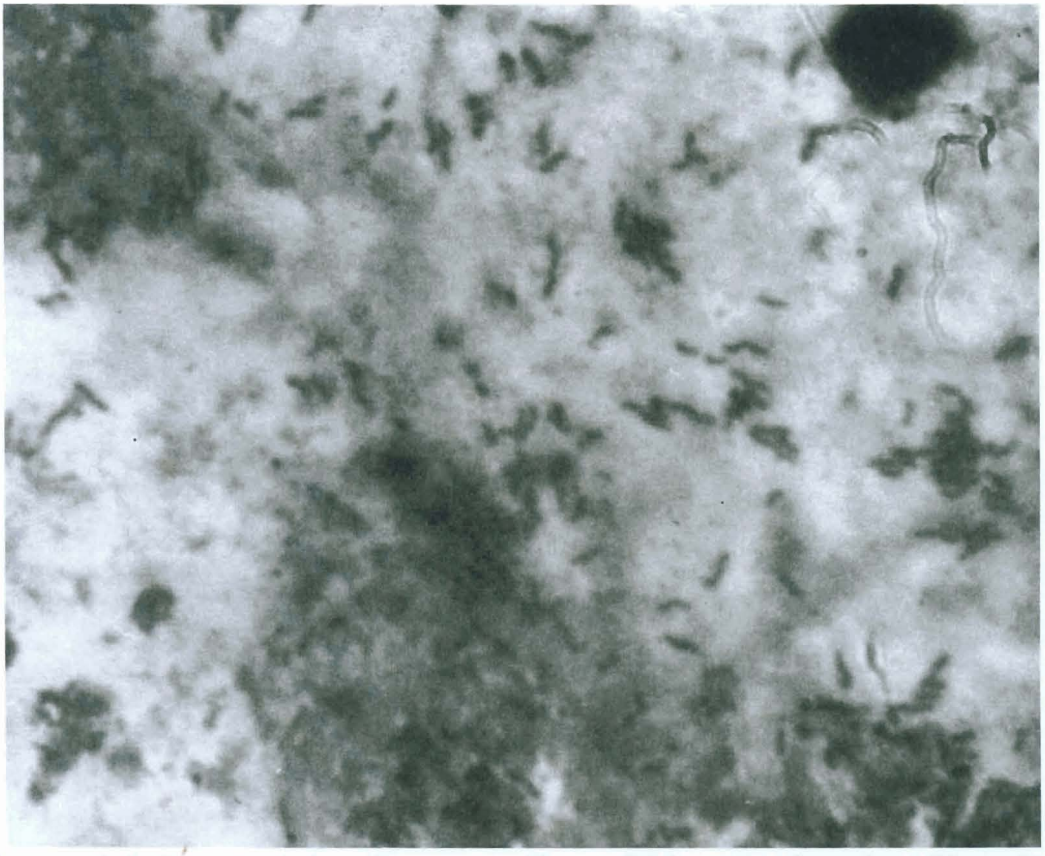

Figure 3.1 Gram stained preparation of biopsy showing *H. pylori*

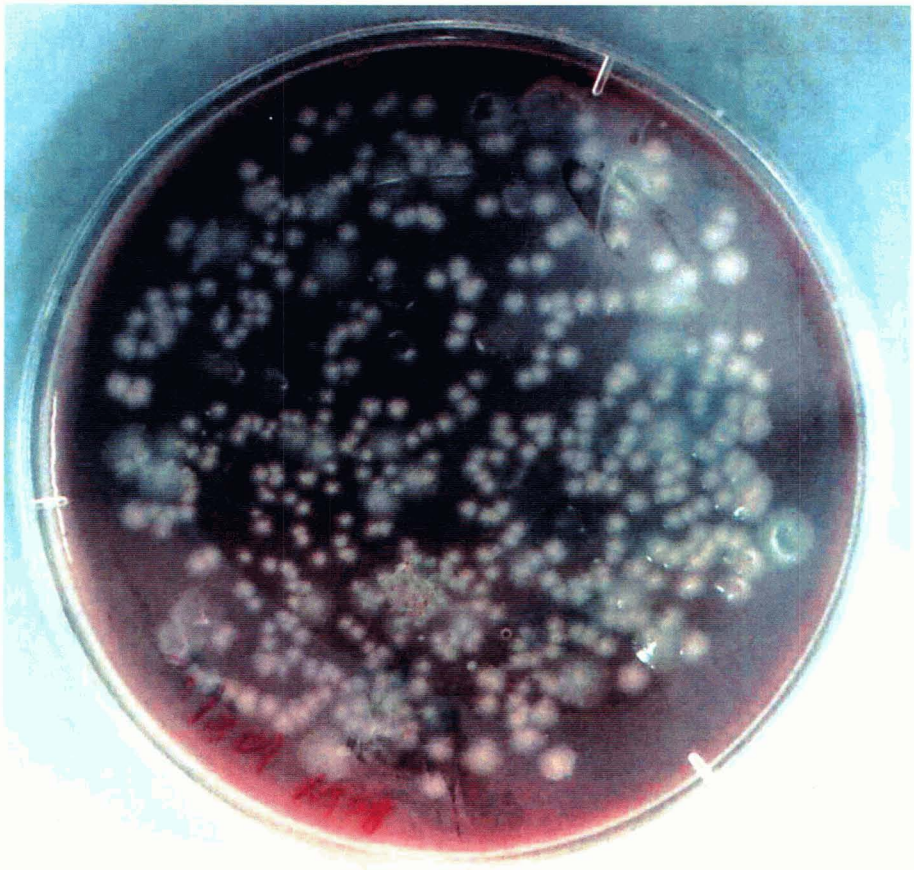

Figure 3.2 Colonies of *H. pylori* on chocolate agar

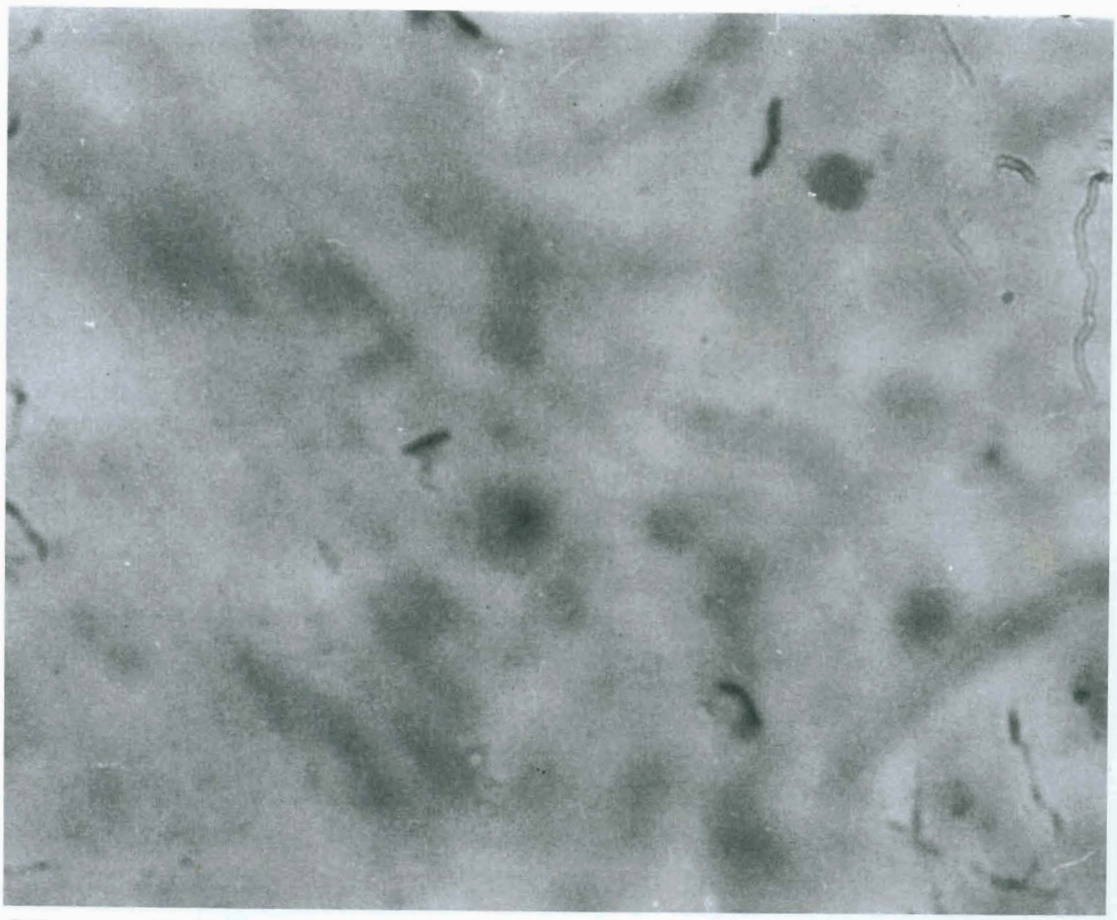

Figure 3.3 Gram stained smear of colonies of *H. pylori*

colonies showed organisms similar to that of organisms present in biopsy samples. It may be noted that these colonies were isolated from the biopsies, which showed the presence of *H. pylori.*

Among the 12 isolates 4 were obtained from duodenal ulcer cases, 4 from healing duodenal ulcer, 3 from duodenitis and one from gastritis. Each isolates were stored in Brucella broth with foetal calf serum (20%) and glycerol (30%) at $^{-}$20°C.

Table 3.3 Gram's staining and biochemical characters of isolates identified as *H. pylori.*

Character	Reaction
Gram's staining	Gram-negative, slightly curved rods
Motility	Motile
Urease test	Strongly Positive
Catalase test	Strongly Positive
Oxidase test	Strongly Positive
Ability to grow aerobically	No growth
Glucose utilization	Negative
Nitrate reduction	Negative

DISCUSSION

The majority of *H. pylori* organisms are free living in the mucus layer of the stomach overlaying the mucosal epithelium, a small proportion appear to adhere epithelial cells, and few, if any, actually invade tissue (Blaser and Parsonnet, 1994). *H. pylori* population is maintained at high concentration (about10^8 CFU/g) in gastric tissue for decades. But the distribution of the organism is not uniform and mostly patchy distribution was observed. So the sampling error could be leading to fallacious results.

According to Misra et al. (2000), two biopsies taken from A_3 region of the antrum were sufficient for confirmation of the presence of *H. pylori* and associated gastritis. These biopsies should be taken from the most normal appearing mucosa (Graham and Qureshi, 2001).

Successful isolation of *H. pylori* from gastric biopsies relies on several factors, such as the transport medium, time in transport to the laboratory, temperature during transportation and medium used for isolation. Several media were recommended for transportation of the specimen. Normal saline or 20% glucose (Goodwin et al., 1985), Thioglycollate broth (von Wulffen et al., 1986; Queiroz et al., 1987), Brucella broth (Taylor, 1988), Carry-Blair medium (Kawanashi et al., 1995; Nakao et al., 1997), etc. were some of the common transport media used.

The temperature during transportation highly influences the viability of *H. pylori*. There was a significant decrease in viability of organism after 6 h at room temperature. At 4°C the organism survived for one week and at $^{-}$70°C, or in liquid nitrogen, it will survive indefinitely (Graham and Qureshi, 2001). It was recommended to transport biopsy samples in transport medium at 4°C and processed within 4-5 h, if in glucose or saline (Rautelin and Kousunen, 1991).

H. pylori can be readily identified on the basis of characteristic colonial and microscopic morphology, a positive oxidase and catalase test, and the rapid hydrolysis of urea. Many authors solely depended on these characters to identify the organism in primary isolation (McNulty et al., 1989; Lin et al., 1992; Nishiya et al., 1999).

Different methods are used for the diagnosis of *H. pylori* infection. Histology provides marked evidence for *H. pylori* infection. *H. pylori* cells were present in sufficient quantities to be seen with high-power oil-immersion magnification of histologic sections or of Gram stains of smears of gastric mucus (Graham and Qureshi, 2001).

Based on the ability of the organism to produce large amount of urease, both non-invasive (urea breath tests) and invasive (rapid urease tests) tests were developed to diagnose *H. pylori* infection. Atherton and Spiller (1994) gave an overall view and importance of urea breath test for diagnosis of *H. pylori* infection. ^{13}C-UBT gave reliable information about *H. pylori* status of the patient before or after therapy (Klein and Graham, 1993; Klein et al., 1996). Rapid urease test is widely accepted and it showed almost 100% specificity when compared with detection of *H. pylori* by Gram staining, culture and histology (McNulty et al., 1989).

HpSA is another non-invasive test developed and found to be easy-to-use test for diagnosing *H. pylori* infections (Vaira et al., 1999). Combined with UBT, HpSA can be used for confirmation of *H. pylori* eradication.

PCR techniques are valuable for diagnosing *H. pylori* infection. In a comparative study, van Doorn et al. (2000) observed that PCR had highest diagnostic sensitivity followed by histology, culture and CLO tests. At the present time PCR remains a research tool for the diagnosis of *H. pylori* infection.

The isolation of the organism in the culture is 100% specific. The sensitivity of the culture varies from 50-99% depending on the laboratory and interest of the microbiologist (Graham and Qureshi, 2001).

The gastric ulcers were considered as an infectious disease and recommended therapy to switch from treating symptoms with antacids to antibiotic eradication of the infection (NIH consensus statement, 1994). But many treatment strategies failed to eradicate *H. pylori* from patients. This was mainly due to primary and acquired resistance of *H. pylori* strains to antimicrobial agents. Glupczynski and European multicentre study group on antibiotic susceptibility to *H. pylori* (1992) observed an overall resistance of metronidazole 26% in European countries, which ranged from 7% in Spain to 49% in Greece. Metronidazole resistance in France was 25%, but it increased rapidly reaching 50% in 1994 (Megraud et al., 1994). Abraham et al. (1997)

reported over ⅔ of all strains isolated from Lucknow and Mumbai were metronidazole resistant. About 90% strains from Kolkata were metronidazole resistant (Mukhopadhyay et al., 2000).

The primary resistance to macrolides was much lower than that for metronidazole. Westblom and Unge (1992) observed less than 1% macrolide resistance. Wolle et al. (2002) reported 26.2% metronidazole resistance and 2.25% clarithromycin resistance in *H. pylori* strains isolated from North Eastern part of Germany.

Coexistence of metronidazole resistant and sensitive strains in patients with peptic ulcer and gastritis were reported (Loivukene et al., 2000). Among metronidazole resistant strains, combined resistance to clarithromycin, ciprofloxacin, and tetracycline was reported (Boyanova et al., 2000). van der Ende et al. (2001) showed the co-existence of clarithromycin resistant and sensitive strains with identical genotypes.

In this circumstance, it was suggested to test *H. pylori* susceptibility to antimicrobial agents and interpretation of the results before commencing antimicrobial therapy. Isolation of the strains from each patient is the only diagnostic procedure, which provides samples for antimicrobial susceptibility testing. More over no reports were available from Kerala showing the prevalence of *H. pylori* infection and successful isolation of the organism from patients. The above facts led to standardize the method of isolation of *H. pylori* from human gastric biopsy specimens and use them for further immunological studies.

The present investigation showed that about 54% of persons with different gastroduodenal symptoms were positive for *H. pylori* infection, as assessed by using rapid urease test (RUT), in and around Kozhikode District of Kerala State. Rapid urease test is one of the reliable invasive diagnostic tests for *H. pylori* infection. According to Lin et al. (1992) biopsy of the antrum at gastroscopy and rapid urease test were most useful for the initial diagnosis of *H. pylori* infection. The sensitivity and

specificity of different urease tests ranged from 95 and 100% respectively (El-Zimaity et al., 1995).

Rapid urease test requires a high bacterial density in the specimen. The photographs of the biopsy specimen show the presence of microorganism abundantly and because it is an ulcer specimen, it was speculated that these organisms are mainly *H. pylori*. The rapid urease test with these biopsies was strongly positive. Of the biopsies used for isolation of *H. pylori*, there were more samples from healing duodenal ulcer patients. Only 44% of these samples were positive for rapid urease test and could isolate only four *H. pylori* colonies. This could be explained as the patients were under anti-ulcer therapy and because the infection was brought under control by eradicating the organism from the site of infection.

In the present study the *H. pylori* colonies were developed only from those biopsy samples, which were positive for rapid urease test. Analysis of the morphological and biochemical characters along with standard strain obtained from Dr. A. Grob (Germany), confirms the identity of the isolates. Many authors applied colony characters, morphology and biochemical characters as criteria for identification of *H. pylori* (Hu et al., 1992; Yang et al., 1997; Loivukene et al., 2000).

Primary isolation of *H. pylori* from biopsy specimen is a difficult process. In specialized laboratories the isolation rates of 75-90% were achieved (Goodwin et al., 1985). This may be due to the fastidious nature of *H. pylori* and to a number of factors that are difficult to control, like patchy distribution of the organism on the gastric mucosa, contamination of biopsy forceps, presence of oropharyngeal flora, loss of viability of organism during transportation (Piccolomini et al., 1997).

Transport medium, time for transportation and temperature at transportation highly influences isolation of *H. pylori*. However, according to Morton and Bardhan (1995) transport medium used is irrelevant provided biopsy specimen can be rapidly transported for culture. They obtained 56% and 50% isolation rates when the specimen

cultured within 3 h after transportation in complex media or 0.9% saline respectively. A long transportation time decreases the number of the *H. pylori* especially after antibiotic therapy, and if the number of bacteria is low, culture may become false negative (Andersen and Wadstrom, 2001).

The major factors responsible for transformation of spiral shaped *H. pylori* into coccoid forms are nutrient deprivation, exposure to anti-ulcer drugs and antibiotics, extended incubation, pH, and attachment to the gastric epithelium (Andersen and Wadstrom, 2001). The coccoid forms are the non-culturable, senescent forms of *H. pylori*. Minimizing the subculturing can minimize the transformation of *H. pylori* into coccoid forms. The isolates were prepared in aliquots and stored at $^{-}$20°C.

Culturing on solid medium is the standard technique used in most laboratories for the isolation of *H. pylori* from gastric biopsies. 12 isolates from 22 rapid urease test positive biopsies were isolated in the present study. The rate of isolation is about 55%. Gastric biopsies taken during endoscopy have been reported as the best samples for the isolation of the *H. pylori* from patients (Rautelin and Kousunen, 1991). The culture results obtained from gastric biopsies were highly dependent upon the accuracy of the biopsy sampling and the bacterial load in the tissue sample (Mobley et al., 1988).

Thus, from the morphological and biochemical characters, it was concluded that the isolates obtained from biopsy specimens were *H. pylori*. Approximately 50% of the ulcers are at least due to *H. pylori* infection.

Chapter IV

***IN VITRO* PROLIFERATION AND IgG SUBCLASS SECRETION IN RESPONSE TO *HELICOBACTER PYLORI* ANTIGENS**

CHAPTER IV

IN VITRO PROLIFERATION AND IgG SUBCLASS SECRETION IN RESPONSE TO *HELICOBACTER PYLORI* ANTIGENS

The immune system of gastric and intestinal mucosa has the daunting task of co-existing with an incredibly complex mix of luminal antigens, including partially digested dietary constituents, host proteins, and commensal bacteria, while maintaining the capacity to recognize and eliminate promptly pathogenic microorganisms and transformed epithelial cells. The mucosal immune system is ideally adapted to recognize and eliminate invading pathogens rapidly, while not responding to ubiquitous luminal antigens. Under normal conditions an active state of tolerance is maintained in gastrointestinal tract. Tolerance is mediated by regulatory T cells and by early detection of invading pathogens by epithelial cells that liberate chemotactic signals to incite immigration of effector cells, which are more efficient in clearing infections (Sartor, 2002).

Uninfected stomach does not show the evidence of immunological activity. Infection with *H. pylori* delivers powerful stimuli that profoundly restructure gastric cell composition and signaling, which re-programme the stomach to function as a target end organ for leukocyte homing and amplifies the immunologic machinery to buttress local T-cell activation during infection (Papo et al., 2001). Lymphocyte homing behaviour was similar in stomach and intestine (Jarbrink et al., 2001). The authors observed the expression of mucosal homing receptor integrin $\alpha_4\beta_7$ on both B-cells and T-cells activated by antigens delivered to the gastric mucosa, as do cells activated in the intestine.

Between 60% and 80% of the body's immunoglobulin secreting cells are found within mucosal surfaces, and most are present in gastro-intestine associated lymphoid tissue (Sartor, 2002). Mattson et al. (1998) demonstrated induction of strong antibody

responses in human gastric mucosa infected with *H. pylori*. The response was observed both in asymptomatic carriers and duodenal ulcer patients.

H. pylori lead to progressive accumulation of T-cells in the proximal and distal stomach. These infiltrated T-cells were predominantly $CD4^+$ Th cell phenotype (Bamford et al., 1998). Karttunen et al. (1995) observed higher number of IFN-γ secreting cells per 10^5 T-cells in gastritis mucosa than in normal mucosa. The over abundance of IFN-γ^+ cells and IFN-γ secreting cells relative to IL-4 producing cells were observed in *H. pylori* positive gastric biopsies (Bamford et al., 1998). Thus, there is predominance of pro-inflammatory Th1 type cells in *H. pylori* infected stomach.

The results obtained by Karttunen et al. (1990) showed that peripheral blood mononuclear cells can be stimulated by whole *H. pylori* bacterium. The response of low-grade B-cell MALT lymphomas to stimulating strains of *H. pylori* was dependent on *H. pylori* specific T-cells and their products, rather than the bacteria themselves (Hussell et al., 1993).

Both local and systemic humoral responses were induced against *H. pylori*. Crabtree et al. (1993) demonstrated the presence of anti-*H. pylori* IgG in serum and anti-*H. pylori* IgA in mucosa of gastric cancer patients by using ELISA and immunoblotting. Some studies revealed significantly higher levels of salivary anti-*H. pylori* IgG in children with *H. pylori* infection (Luzza et al., 1997). Even though detectable levels of *H. pylori* specific IgA present in serum samples of patients, it has low diagnostic value (Karvar et al., 1997).

Seropositivity of IgG antibody against *H. pylori* correlated with positivity of tissue *H. pylori* IgA antibody in non-metaplastic gastric mucosa (Matsukura et al., 1995). Anti-*H. pylori* IgM titres were very low in patient sera and it was not related to

histopathological findings, peptic lesions or culture findings (von Wulffen and Grote, 1988). This may be due to the chronicity of *H. pylori* infection.

In IgG immunoblots, four protein bands (110 kD, 94 kD, 63 kD, and 28 kD) were consistently observed in *H. pylori* positive patients (von Wulffen et al., 1988). The authors were of the opinion that the IgM immunoblots were of no help in diagnosis of *H. pylori* infection because the only band recognized in IgM immunoblot was non-specific. IgG immunoblot with *H. pylori* positive children recognized 120 kD, 90 kD, 61 kD, 53 kD, 45 kD, and 31 kD protein bands (Crabtree et al., 1991). It was found that the initial response to *H. pylori* infections in children were directed to low molecular weight antigens and in adults the initial response was to high molecular weight antigens (Mitchell et al., 1996).

Stacey et al. (1990) demonstrated the presence of antibodies against *H. pylori* urease and flagellins in patient sera. Immunization with *H. pylori* catalase protected the animals from experimental infection with *H. pylori* (Radcliff et al., 1997).

A predominant antigenic structure present in *H. pylori* is lipopolysaccharides. Zheng et al. (2000) observed the occurrence of Le^x, Le^y, Le^a, Le^b and blood group A antigens in *H. pylori* isolates. The authors also noted the co expression of Le^a with Le^b. There is definitive evidence showing a single strain of *H. pylori* alters its LPS antigenic phenotype during the course of infection (Rasko et al., 2000). Serum anti-Le^x antibodies were detected in patient sera but it was not increased significantly in *H. pylori* positive gastric cancer patient serum samples (Shimoyama et al., 2000).

Several antigens were tried to use as an effective vaccine against *H. pylori* infection. Marchetti et al. (1995) used total bacterial lysate or purified urease of *H. pylori* for oral immunization of mice along with heat labile toxin of enterotoxigenic *E. coli* as adjuvant. When immunized mice were challenged with Type II strains of *H. pylori*, complete protection was observed with total bacterial lysate and almost complete protection was

afforded by immunization with urease. Manetti et al (1997) detoxified VacA and used for immunizing rabbit and mice. High titres of cytotoxin neutralizing antibodies were induced in immunized rabbits and challenge with *H. pylori* in immunized mice showed protection against infection. *H. pylori* catalase was also proposed as candidate vaccine against infection (Radcliff et al., 1997).

Human immunoglobulin G comprises four subclasses depending on their heavy chain regions (IgG_1, IgG_2, IgG_3, IgG_4). Each of these subclasses showed difference in their biological functions. Normansell (1987) reviewed the structural and functional properties of human IgG subclasses.

The expression of IgG subclasses depends on type of antigens inducing the response. The bacterial protein antigens and viral antigens induce IgG_1 and IgG_3 subclasses predominantly (Hammerstrom and Smith, 1986; Samesto et al., 1985). The response to bacterial polysaccharides largely restricted to IgG_2, to IgG_2 and IgG_4, or to IgG_1 and IgG_2 (Hammerstrom and Smith, 1986). Raised IgG_4 serum levels were reported in persons with atopy and filariasis (Magnusson et al., 1986) and in Schistosomiasis (Iskandar et al., 1981).

Several assay methods are used for the quantitation of human IgG subclasses. Radial immunodiffusion method of Mancini (Shakib et al., 1975), Radioimmuno assay (Scott and Nahm, 1984), Particle concentration fluorescence immunoassay (Mayus et al., 1986) and ELISA (Ferrante et al., 1986) were some examples. Monoclonal antibody based ELISA technique is the most commonly used method for assaying IgG subclasses.

The second objective of the present investigation was to isolate *H. pylori* antigens following different methods and to examine the *in vitro* lymphocyte proliferation and antibody production in response to these antigen stimulations.

RESULTS

The protein profiles of the different antigen preparations were analyzed by SDS-PAGE. The Figures 4.1 to 4.3 show the pattern of protein bands present in acid-glycine (AG), n-Octyl-β-D-glucopyranoside (NOG) and whole cell sonicate (WCS) antigens obtained from different clinically isolated strains of *H. pylori*, respectively.

Heavy molecular weight protein bands were distinctly present in most of the n-octyl-β-D-glucopyranoside extract preparations. The major bands present in n-octyl-β-D-glucopyranoside preparations showed approximate molecular weights 120 kD, 92 kD, 74 kD, 66 kD, 50 kD, 26 kD, 24 kD, and 14.8 kD.

Acid-glycine preparations of all isolates showed mainly low molecular weight protein bands. Major bands observed in these preparations fall in the range of approximate molecular weight 50-14.3 kD. 4-5 distinct minor bands were present at the range of 68-50 kD. Although high molecular weight proteins of 120 kD and 92 kD were present in acid-glycine extract, the intensity of these bands was very low. This indicates that acid-glycine mainly extract proteins of low molecular weight.

The major protein bands obtained in whole cell sonicate preparations were in the range of 74-14.3 kD. Isolates 3 and 9 did not show any bands above 74 kD. Isolate 5 showed a distinct band of low intensity corresponding to 120 kD and 3-4 minor bands ranging from 68-97 kD. Most of the low molecular weight bands present in other preparations were also present in whole cell sonicate preparations from all isolates.

No detectable level of proteins was present in hot phenol water extracts from clinical isolates as assessed by Lowry's method. SDS-PAGE analysis of these preparations could not detect any detectable bands with coomassie blue staining.

The antigens extracted from isolated *H. pylori* strains were used as antigens to stimulate the tonsillar mononuclear cells. The proliferation was assessed using MTT assay.

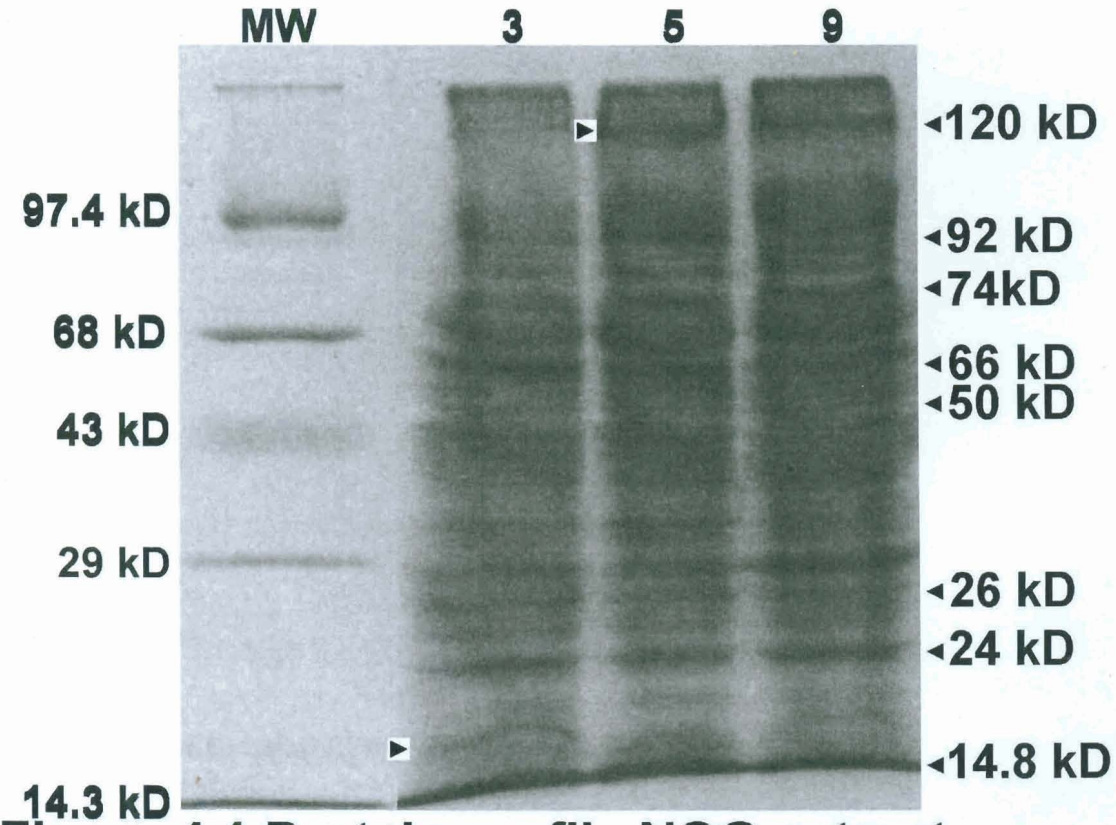

Figure 4.1 Protein profile NOG extract antingens of strain 3, 5, and 9

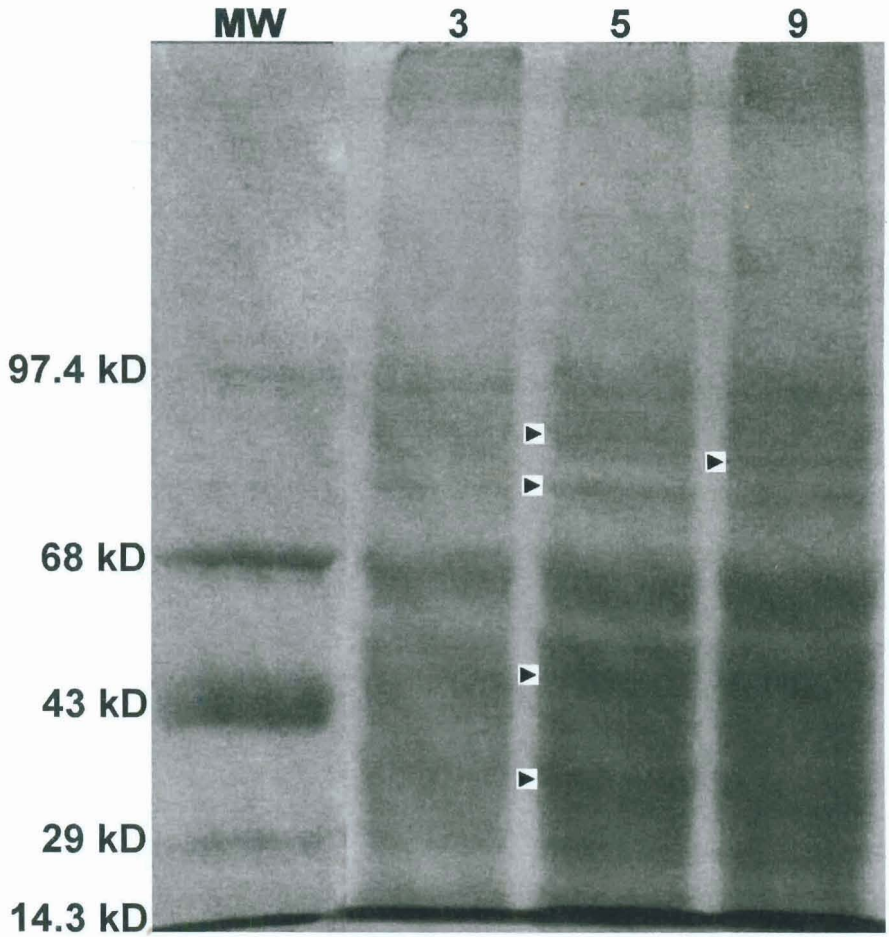

Figure 4.2 Protein profile of acid-glycine extract antigens of strains 3, 5, and 9

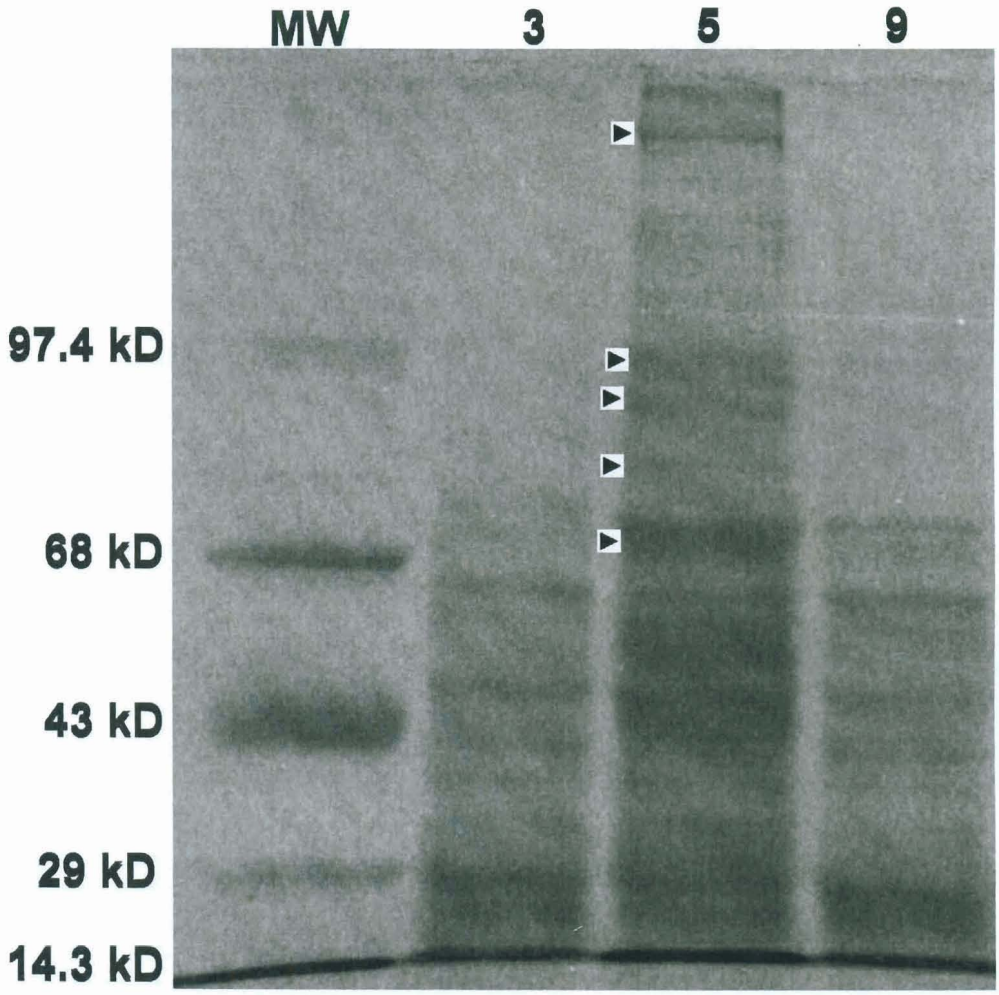

Figure 4.3 Protein profile of WCS extract antigens of strain 3, 5, and 9

PHA and ConA were included as known mitogens in the experimental protocol. There was no significant increase in proliferation with *H. pylori* antigens. Maximum proliferation index was obtained with mitogens. Among the antigens, whole cell sonicate preparations yielded least proliferation of tonsillar mononuclear cells (Table 4.1). Since 10 µg/ml gave the maximum proliferation index, this concentration was chosen for further studies of *in vitro* secretion of IgG subclasses.

Table 4.1 Proliferation indexes obtained with various *H. pylori* antigens and mitogens

Antigen/Mitogen 10 µg/ml	Proliferation index
LPS3	0.9225
LPS5	0.9275
LPS9	0.9277
AG3	0.9237
AG5	1.0871
AG9	0.9585
NOG3	0.8156
NOG5	1.0269
NOG9	0.9642
WCS3	0.8989
WCS5	0.7897
WCS9	0.7533
Con A	1.1480
PHAM	1.5914

The level of IgG subclasses in the culture supernatant was analyzed after 7 days growth of tonsillar mononuclear cells with different antigens and mitogens. The concentration of IgG subclasses was 2.7 µg/ml IgG_1, 1.24 µg/ml IgG_2, 0.41 µg/ml IgG_3 and 0.136 µg/ml IgG_4 in control supernatant.

An increase in the secretion of total IgG by tonsillar lymphocytes was noted with PHA (18.6 µg/ml). Among the different IgG subclasses IgG_2 is the major subclass induced in response to PHA stimulation (11.6 µg/ml). It also induced 4.4 µg/ml IgG_1, 1.72 µg/ml IgG_3 and 0.88 µg/ml IgG_4 (Table 4.2)

In contrast ConA did not increased the secretion of IgG subclasses. In fact the ConA stimulated cultures produced lower level of IgG subclasses when compared to the control. From the data it can be observed that ConA has no stimulatory role in the IgG subclass secretion.

When different antigen extracts were used for induction of IgG subclasses, it was noted that the maximum level of total IgG production was obtained with n-octyl-β-D-glucopyranoside extracted antigens and that too with that of the isolate strain No.5. The least amount of total IgG was noted when whole cell sonicate antigen from isolate strain No.5 was used (Table 4.2).

Among the various antigens of phenol-water extracts, the maximum IgG production was observed with isolate strain No. 3 (Table 4.2) and the production of IgG_2 subclass was higher than the control.

With acid-glycine extracted antigens it was noted that total IgG was maximally produced with isolate strain No. 5 and least production with isolate strain No. 3. On further analysis it was observed that the IgG_1 was the major subclass present in the IgG secreted in presence of the acid-glycine extracted antigens from all three isolates.

Table 4.2 IgG subclass secretion in response to *H. pylori* antigens and mitogens

Antigens/ mitogens	IgG_1 (µg/ml)	IgG_2 (µg/ml)	IgG_3 (µg/ml)	IgG_4 (µg/ml)	Total IgG (µg/ml)
Control	2.7	1.24	0.41	0.136	4.486
ConA	2.5	0.37	0.59	0.24	3.7
PHAM	4.4	11.6	1.72	0.88	18.6
LPS3	3.2	2.46	0.46	0.36	6.48
LPS5	2.0	3.2	0.152	0.234	5.586
LPS9	2.1	3.2	0.154	0.234	5.688
AG3	1.56	0.76	0.132	0.116	2.568
AG5	2.6	0.59	1.56	0.3	5.05
AG9	1.68	1.28	1.204	0.152	4.316
NOG3	1.0	0.6	0.26	0.018	1.878
NOG5	1.68	3.6	9.4	0.44	15.12
NOG9	3.0	1.52	1.04	0.062	5.622
WCS3	1.16	0.108	0.29	0.092	1.65
WCS5	0.7	0.074	0.28	0.116	1.17
WCS9	2.34	0.106	0.56	0.185	3.191

n-octyl-β-D-glucopyranoside extracted antigens showed higher production of total IgG, except isolate strain No. 3. The protein profile of the n-octyl-β-D-glucopyranoside extract of isolate strain No. 3 showed the absence of high molecular weight protein bands in comparison with isolate strain No. 5 and 9 (Figure 4.1). The antigens induced the production of higher levels of IgG_3, except with isolate strain No. 3. A preferential secretion of IgG_3 was noted with isolate strain No. 5 and this was almost 22 times higher in comparison to the control. However, isolate strain No. 3 yielded lesser IgG_3 than the control.

Whole cell sonicate antigens did not enhance IgG production. It may be noted that whole cell sonicate antigens produced least proliferation index when compared to other antigen preparations (Table 4.1). The failure of whole cell sonicate antigens to induce the secretion of IgG subclasses may be due to the presence of inhibitory molecules in the whole cell sonicates.

When the percentage of secretion of IgG subclasses *in vitro* by tonsillar mononuclear cells was analysed, it was found that IgG_1, IgG_2, IgG_3, and IgG_4 constituted 66.26%, 27.67%, 9.13% and 3.03%, respectively in control wells. Among different antigen extracts, only whole cell sonicate antigens of isolate strain No. 3 and 9 and acid-glycine extracted antigen of isolate strain No.3 showed increased percentage in IgG_1 secretion when compared to the control. Increased percentage of secretion of IgG_2 was observed with all phenol-water extracted antigens. The percentage of secretion of IgG_2 was significantly lower in presence of all whole cell sonicate antigens (Figure 4.4). The percentage of secretion of IgG_3 was higher in stimulation with all antigen preparations, except phenol-water extract antigens from all isolates and acid-glycine extract antigens from isolate strain No. 3.

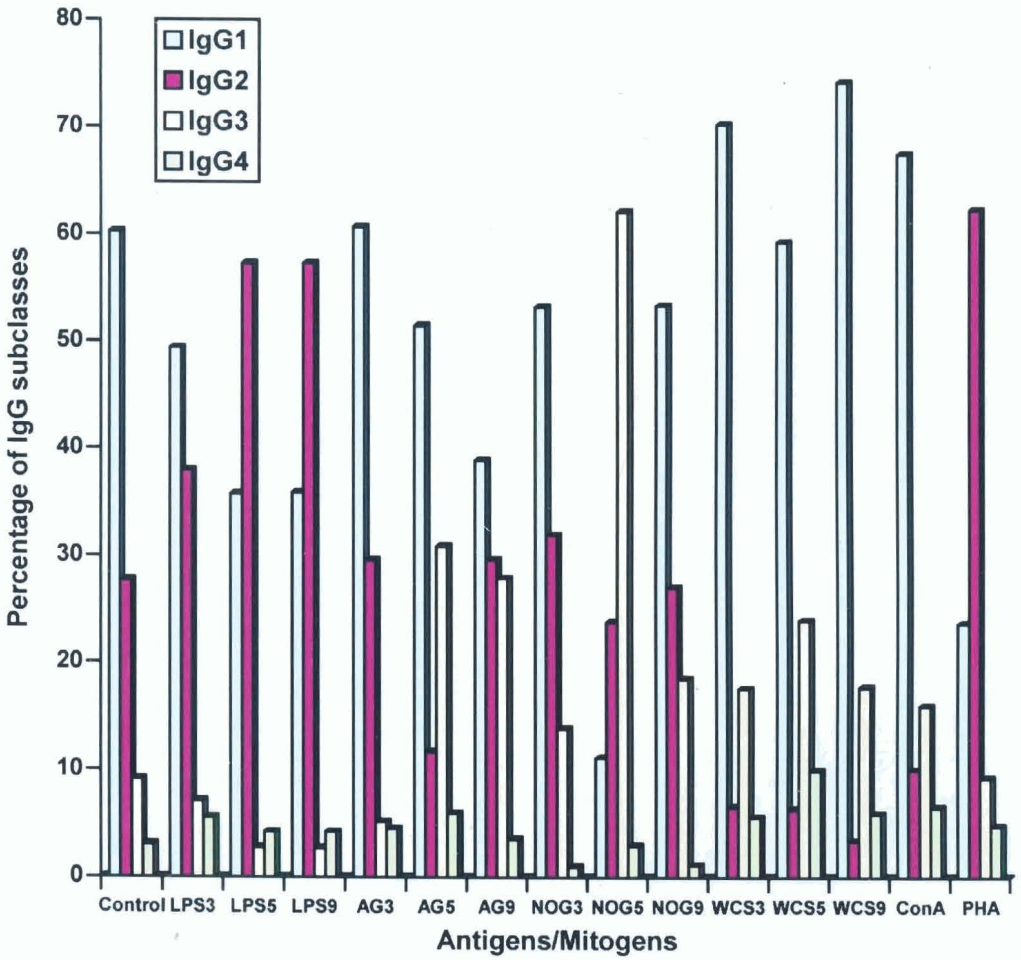

Figure 4.4 Percentage of IgG subclasses secreted in response to the stimulation with *H. pylori* antigens/mitogens [LPS (Phenol water extract), AG (Acid-glycine extract), NOG (n-octyl-β-D-glucopyranoside extract), and WCS (Whole cell sonicate) preparations of strains 3, 5, and 9]

DISCUSSION

Several authors analyzed antibody levels against specific components of *H. pylori* cells. Klaamas et al. (1996) observed antibodies against VacA and CagA proteins in immunoblots with sera of *H. pylori* positive gastric cancer patients. Hirai et al. (1994) reported a strong association of cytotoxin neutralizing activity with *H. pylori* infection. IgG antibodies to VacA and CagA proteins were observed in 85% of patients with gastric symptoms and 71% of asymptomatic subjects (Figueroa et al., 2002). Anti-CagA IgG was detected in 87.5%, 76%, and 56.4% of duodenal ulcer, gastric ulcer, and non-ulcer dyspepsia patients (Cover et al., 1995). Kumar et al. (1998) demonstrated the prevalence of anti-CagA antibodies in an Indian population with duodenal ulcer or non-ulcer dyspepsia.

O'Toole et al. (1991) reported a 26 kD soluble protein from *H. pylori* as species-specific antigens. This protein appeared antigenically conserved in *H. pylori*. Keenan et al. (2000) observed immune response to an 18 kD outer membrane protein in a significant number of immunized animals. This protein was subsequently identified as Lipoprotein 20 (Lpp 20).

The detection of anti-*H. pylori* antibodies are the easiest non-invasive approach to test for the presence of *H. pylori* infection. These tests detect either IgG (Klaamas et al., 1996; Asaka et al., 1994) or IgA (Jaskowski et al., 1997; Kindermann et al., 2001) in serum. Tests, which detect the presence of anti-*H. pylori* antibodies in saliva were available (Luzza et al., 1997). Most of the tests used enzyme immunoassays. These assays used different antigen preparations from *H. pylori* strains, such as acid-glycine extracts (Klaamas et al., 1996; von Wulffen and Grote, 1998) HM-CAP obtained by n-Octyl- β-D- glucopyranoside extraction (Evans Jr. et al., 1989; Yokota et al., 1997). Purified antigens like recombinant CagA protein (Cover et al., 1995), immunodominant

portion of CagA (Kumar et al., 1998), Le^x oligosaccharide (Shimoyama et al., 2000) were also used for the detection of immune response against specific antigens of *H. pylori*. For immunoblot analysis of patient sera, several authors used whole cell lysate antigens (von Wulffen et al., 1988; Crabtree et al., 1991).

The diagnosis of *H. pylori* infection is normally done either using rapid urease test or with anti-*H. pylori* IgG ELISA tests. Commonly acid-glycine extract antigens (Klaamas et al., 1996), high molecular weight cell associated protein (HM-CAP) antigens (Evans Jr. et al., 1989) from *H. pylori* were used in diagnostic kits. Several immunoblot studies aimed to analyze immune response against *H. pylori* utilized whole cell sonicate antigens (Crabtree et al., 1991; von Wulffen et al., 1988). A considerable amount of variability in the detection of anti-*H. pylori* IgG antibodies were reported when these antigens based ELISA was employed (Kinderman et al., 2001; Meijer et al., 1997). These variabilities in the sensitivity and specificity were attributed primarily to the differences in types of *H. pylori* antigens used for coating the microtitre plates. In this context, the determination of *in vitro* cell proliferation and IgG subclass secretion by human mononuclear cells in stimulation with the antigens isolated from different clinical isolates of *H. pylori* became one of the aims of the present study.

Antigens from clinical isolates of *H. pylori* strains (Nos. 3, 5 and 9) were prepared by using different methods as described in materials and methods and used for *in vitro* proliferation and IgG secretion by human tonsillar mononuclear cells. The analysis of protein profiles of the antigen preparations from *H. pylori* clinical isolates by SDS-PAGE showed strain variations. The acid-glycine extract showed similar pattern of protein profile in isolate strains 5 and 9 differing from isolate strain No. 3. Two protein bands corresponding to approximate molecular weights 32 kD and 50 kD in isolate strains 5 and 9 were obviously absent in isolate strain 3. When isolate strains 5 and 9 were compared, a protein band of molecular weight 82 kD was absent in isolate strain No. 5. When IgG

subclass secretions in presence of acid-glycine extract antigens were compared isolate strain 3 induced least amount of secretion (2.568 µg/ml) as compared to the other two isolates (5.05 µg/ml and 4.316 µg/ml with isolate strains 5 and 9, respectively). Although no attempts were made to purify these two protein fractions and to examine its impact on *in vitro* IgG subclass secretion, it is speculated that such proteins may be playing important role in priming the cells to produce immunoglobulins. However, no major band of high molecular weight proteins above 97 kD was obtained with acid-glycine extraction.

When the protein profiles of n-octyl-β-D-glucopyranoside extract antigens were compared, it was seen that the isolate strain No. 3 exhibited a different pattern compared to the isolate strains 5 and 9. A significant high molecular weight band (about 120 kD) was absent in isolate strain No.3. When the protein profiles between the isolate strains 5 and 9 were compared, it was noted that a low molecular weight fraction corresponding to molecular weight of about 15 kD is absent in isolate strain No. 9. Perhaps this may be the reason for higher secretion of IgG by the isolate strain No. 5 (15.12 µg/ml) compared to isolate strain No. 9 (5.622 µg/ml). The n-octyl-β-D-glucopyranoside extract antigens yielded defined bands of high molecular weight, i.e., proteins of molecular weight above 66 kD. These observations are in agreement with the observations of Evans Jr. et al. (1989).

When *in vitro* proliferation of human tonsillar mononuclear cells were analyzed by using MTT assay, the significant increase in proliferation index was obtained with mitogens (PHA and Con A) only. In presence of different antigens of *H. pylori* strains, no increase in proliferation was noted. Knipp et.al. (1996) reported the production of protein by *H. pylori.* which inhibits cell proliferation. Recently inhibition of T-cell activation by *H. pylori* VacA was reported (Gebert et al., 2003). A G1/S cell cycle arrest was observed in inhibited T-cells. Bonesrestiano et al. (2003) also reported similar results.

In the present study, the protein profiles of used antigens showed a large number of proteins of different molecular weights that may also contain VacA and other proteins, such as the proteins described by Knipp et al. (1996) - the well-known inhibitors of cell proliferation. T-cell activation and subsequent release of cytokines are required for the B-cell activation, proliferation and differentiation into antibody producing cells in T-dependent pathway. Perhaps this may be the reason that none of the protein antigen preparations, except acid-glycine extract and n-octyl-β-D-glucopyranoside extract antigens from isolate strain No. 5, showed increased proliferation and antibody production. It may also be noted that most of the tonsillar cells, especially B-cells are at activated stage, so specific mitogens only can induce further activation as noted in the case of ConA and PHA.

One of the predominant groups of antigens on *H. pylori* is lipopolysaccharide. *H. pylori* expresses surface markers, which can mimic the Lewis (Le) blood group antigens of human beings. There are evidences for the contribution of lipopolysaccharide to the pathogenesis of *H. pylori* infection (Slomiany et al., 1992). The antigenicity of *H. pylori* lipopolysaccharides varied depending on the strain (Yokota et al., 1997). Yokota et al. (2000) confirmed the presence of highly antigenic and weakly antigenic epitopes in lipopolysaccharide of *H. pylori* strains. These reports prompted to include hot-phenol water extracts from *H. pylori* isolates for analyzing *in vitro* immunoglobulin secretion.

The phenol-water extract antigens did not induce higher proliferation and IgG secretion. The low immunological activities of *H. pylori* lipopolysaccharides were already reported (Moran and Aspinall, 1998). Muotiala et al. (1992) reported that the pyrogenicity and mitogenicity of *H. pylori* lipopolysaccharide 1000-fold lower in mice when compared with *Salmonella enterica* serovar *Typhimurium* lipopolysaccharide. The induction of interleukin-1 (IL-1), IL-6 and TNF-α from activated human mononuclear cells by *H. pylori* lipopolysaccharide is significantly lower than that by *E. coli* lipopolysaccharide (Birkholz et al., 1993; Pece et al., 1995). But in presence of phenol-water extract antigens the

percentage of IgG_2 subclass was higher when compared to control. This observation is in agreement with general phenomenon that carbohydrate antigens (lipopolysaccharides) selectively induce IgG_2 by following T-independent pathway. Yount et al. (1968) observed that the IgG antibodies produced against dextran, teichoic acid and levan were restricted to IgG_2 subclass. The stimulation of peripheral blood mononuclear cells with lipopolysaccharide resulted in the enhanced production of IgG_2 (Scott and Nahm, 1984). Riesen et al. (1976) noted the IgG_2 restricted response against group specific polysaccharide of beta haemolytic streptococci in human.

Human tonsillar mononuclear cells contain 60-70% B-cells, 30-40% T-cells, 1-8% monocytes and less than 1% dendritic cells. Scott and Nahm (1984) studied *in vitro* IgG subclass production by splenocytes, tonsil cells and peripheral blood lymphocytes in response to mitogen stimulation. All mitogens induced strongest response with splenocytes and least response with peripheral blood lymphocytes. The authors obtained good response with tonsil cells.

Generally, protein antigens stimulate IgG_1 and IgG_3 production and carbohydrate antigens increased IgG_2 production *in vitro* conditions. It was well documented that this pattern of subclass response were mainly against protein antigens of pathogens (Normansell, 1987).

The hot-phenol water extract antigens caused increased production of IgG_2 by tonsillar mononuclear cells when compared to control. Phenol-water extraction procedure was mainly used for extraction of lipopolysaccharides from bacteria (Westphal and Jann, 1965). There are reports showing the preferential expression of IgG_2 subclass in response to LPS or other cell polysaccharides (Mayumi et al., 1983). Siber et al. (1980) noted a positive correlation between serum IgG_2 concentration and the response to pneumococcal polysaccharide antigens.

Among whole cell sonicate antigens; isolate 3 and 9 showed significant increase in IgG_1 and IgG_3 production. The protein profile of these preparations does not show any major bands above 68 kD. Whole cell sonicate antigens from isolate No. 5 showed significant protein bands above 68 kD and it caused higher production of IgG_3 compared to control.

Fangrat et al. (2003) reported IgG_1 and IgG_3 response in children infected with *H. pylori*. Specific proteins, such as CagA also induced IgG_1 and IgG_3 response in these children. No extensive reports are available on the human IgG subclass response against *H. pylori*.

Many immunization studies with different antigen preparations or whole *H. pylori* organism in animal models revealed some aspects of murine IgG subclass expression. Oral immunization of mice with a mixture of whole cell sonicate antigen and cholera toxin induced a greater production of IgG_1 specific to *H. pylori* than IgG_2a in sera of protected mice (Shirai et al., 2000). Vaccination with attenuated *Salmonella enterica* serovar *Typhi* expressing *H. pylori* urease caused an increase in IgG_1 and IgG_2a anti-urease antibody titres in mice (Arcila et al., 2002). Watanabe et al. (2004) demonstrated increasing ratio of IgG_1/IgG_2a in serum of mice following infection with *H. pylori*.

In children, the initial antibody response to *H. pylori* infections were to small molecular size antigens, but in adults the initial responses were to larger molecular size antigens (Mitchell et al., 1996). Karvar et al. (1997) divided the protein antigens of *H. pylori* into 3 major groups depending on the molecular size. Low molecular-mass proteins (LMMPs) comprise proteins ranging in mass from 22-33 kD, medium moleculr-mass proteins (MMMPs) ranging molecular mass from 43-66 kD and high molecular- mass proteins (HMMPs) ranging in molecular mass from 87-128 kD. In their study, 93% of patients showed IgG antibodies against LMMPs and 81% had detectable IgG levels

against HMMPs. Nearly all patients with anti-LMMP antibodies and anti-HMMP antibodies were found to have IgG against MMMPs. Many proteins in MMMPs were proved to be cross-reacting with other pathogens (Johansen et al., 1995).

It is well known that *H. pylori* can effectively modulate the host immune response, which facilitate the chronic persistence of the organism in human host. Immunoassays were widely accepted as simple non-invasive method for diagnosing *H. pylori* infections. IgA detection has no significant diagnostic value (Jaskowski, 1997). IgM titres in patients did not show any correlation with pathological findings (von Wulffen and Grote, 1988). However, IgG immunoassays are widely used in the diagnosis of infection. The wide variations in sensitivity and specificity of different products for detection of anti-*H. pylori* IgG available commercially points for the necessity for identifying IgG inducing immunodominant antigens of *H. pylori*.

H. pylori infection acquire during early childhood (Malaty et al., 2002). Invasive procedures will not be possible in children for diagnosis of *H. pylori* infection. Enzyme immuno assays (EIA) for detection of anti-*H. pylori* antibodies were highly useful in this regard. But the patterns of antigen recognition of children sera were different from that of adults. So the kits for diagnosis of *H. pylori* infection in paediatric population must be evaluated independently.

Thus, from the above study it can be concluded that proteins of *H. pylori* induces IgG_1 and/or IgG_3 subclasses secretion and lipopolysaccharide stimulates the IgG_2 subclass secretion. However, these results cannot be used as indication of *H. pylori* diagnosis.

Chapter V

IMMUNOGLOBULIN G SUBCLASSES IN GASTRIC CANCER PATIENTS

CHAPTER V

IMMUNOGLOBULIN G SUBCLASSES IN GASTRIC CANCER PATIENTS

One half to one third of worlds' population harbour *H. pylori* (Blaser, 1996). The association of *H. pylori* with gastritis was accepted without any dispute. Several authors reviewed the role of *H. pylori* in the pathogenesis of various gastroduodenal conditions (Blaser, 1990; Blaser, 1992; Collins, 1992). Many events caused by *H. pylori* colonization in gastric mucosa alters mucosal integrity, which ultimately lead to inflammation and ulcerations. The major factor include enzymatic and toxic reactions related to *H. pylori* infection, which may further trigger immunological reactions in gastric mucosa and epithelium, as well as the local production of ammonia from endogenous urea, and the production of free radicals (Sipponen, 1992).

H. pylori infection is chronic. The acquisition of the organism in the body occurs during the childhood and the infection risk is quite low in adulthood (Sipponen, 1995). The role of *H. pylori* in chronic gastritis has led to the hypothesis that this infection could be involved in the pathogenesis of gastric cancer (Sipponen, 1994). The main factors that support the hypothesis include: (i) more than 80% of chronic gastritis were caused by *H. pylori* infection, (ii) in a large proportion of infected persons chronic gastritis develop into atrophic gastritis and intestinal metaplasia, (iii) the gastric cancer development risk is high in patients with chronic gastritis and particularly in those with atrophic gastritis. Later studies revealed significantly increased incidences of gastric cancer development in patients with chronic non-atrophic gastritis also (Sipponen et al., 1994). In a prospective study, Uemura et al. (2001) clearly showed the development of gastric cancer in *H. pylori* positive persons.

Satoh et al. (1995) observed more extensive atrophic gastritis in younger age group of *H. pylori* positive patients than *H. pylori* negative patients. Atrophic sequelae of *H. pylori* infection constituted a background factor in the etiology of gastric cancer

(Sakaki et al., 1995). Several reports from various countries showed close association between *H. pylori* infection and its progression to atrophy and intestinal metaplasia, the background lesions for gastric cancer development (Nogueira, et al., 1993; Kuipers et al., 1995; Asaka et al., 1994; Ching and Lam, 1994). These reports supported the suggestion that *H. pylori* as the missing general worldwide environmental factor in the pathogenesis of gastric cancer and in the world epidemiology of gastric cancer (Sipponen, 1992).

Several authors reviewed the associations of *H. pylori* infection with gastric cancer development (Nomura and Stemermann, 1993; Munoz, 1994; Goldstone et al., 1996). Based on the accumulated reports from various regions of the world, IARC/WHO identified *H. pylori* as a group 1 (definitive) carcinogen (Anonymous, 1994).

The hypothesis regarding the association of *H. pylori* infection came from the development of intestinal metaplasia as a result of the pathologic consequence of infection. Many seroprevalence and histological studies proved the association of *H. pylori* infection with gastric cancer (Asaka et al., 1994; Guarner et al., 1993; Nogueira et al., 1993).

H. pylori might be causing gastric carcinoma by converging effects of several events including inflammatory response leading to mutational events in gastric epithelial cells and direct effects on gastric epithelial cell by *H. pylori* organism or released bacterial products.

A 150 kD protein produced by *H. pylori* was found to promote neutrophil infiltration in gastric mucosa and induce production of reactive oxygen radicals (Evans Jr. et al., 1995). ROS (reactive oxygen species) up-regulate IL-8 production in gastric mucosa, which can further promote inflammatory response. *In vitro* studies with live *H. pylori*, culture supernatant and water extracts of *H. pylori* showed significant increase in the production of IL-8 by cultured cells (Yakabi et al., 2000). ROS can

induce DNA damage with the accumulation of mutations, thus contributing to the pathogenesis of gastric cancer (Grisham et al., 2000).

H. pylori infection may be contributing to the increased proliferation of cells in antrum and corpus of gastric epithelium (Lynch et al., 1995a, b). *H. pylori* and bile appear to have a synergistic effect on gastric cell proliferation (Lynch and Axon, 1995). The increased expression of proliferating cell nuclear antigen (PCNA) proved the high epithelial cell turn over in long standing untreated *H. pylori* positive chronic gastritis (Panella et al., 1996) and this could be an indicator of increased risk of neoplastic changes in *H. pylori* infection.

Initially, the analysis of the association of *H. pylori* infection with gastric cancer was based on seroepidemiological studies. Most of these studies compared the level of anti-*H. pylori* antibodies in the serum samples of the patients with gastric cancer and other neoplastic conditions or normal healthy population (Eurogast study group, 1993; Rudi et al., 1995; Asaka et al., 1994)

The expression of specific antigens on cancers depending on the nature of its induction is well documented. Several antigens, such as antigen CA72-4 (Hamazoe et al., 1992), antigen NCC-SG-007 (Kusama et al., 1987) an asialoglycoprotein antigen (Adachi et al., 1987) were also reported to be present on gastric cancer cells.

Humoral immune response have inevitable role in protection of body from infectious diseases and tumours. Several mechanisms of action of antibodies are relevant in this protective role, such as complement activation, opsonization, neutralization of viruses and toxins, induction of ADCC, etc. All these activities of immunoglobulins favour the elimination of antigens from the body.

Human immunoglobulins are a family of glycoproteins that confer humoral immunity and perform vital roles in cellular immunity in man (Goodman, 1987). On the basis of structural, biological and antigenic differences human immunoglobulins were divided into five classes - IgG, IgA, IgM, IgD and IgE. By using polyclonal antisera

prepared against human IgG myeloma proteins, four distinct heavy chain subgroups were identified (Ballieux et al., 1964; Grey and Kunkel, 1964; Terry and Fashey, 1964). Subsequently these subtypes were classified as IgG subclasses 1, 2, 3, and 4 on the basis of their concentrations in normal serum and the frequency of their occurrences as myeloma proteins (WHO report, 1966).

Wide ranges of the concentration of IgG subclasses in normal human sera were reported. Shakib et al. (1975) reported 71.5% IgG_1, 19.4% IgG_2, 8.4% IgG_3 and 0.7% IgG_4 in normal human serum. Papadea and Check (1987) reported the concentration of each subclasses as 1.8-7.8 g/L IgG_1, 1.0-4.6 g/L IgG_2, 0.3-1.4 g/L IgG_3, and 0.08 -1.8 g/L IgG_4 in normal human sera. The IgG and IgG subclass levels in healthy children of various ages were given by Ochs and Wedgewood (1987). Even though there was wide differences in the reported data regarding the levels of IgG subclasses in normal healthy humans, the following general agreement on the actual percentage of the each subclass was accepted: IgG_1 - 60.3-71.5%, IgG_2 -19.4-31.0%, IgG_3 - 5.0-8.4%, and IgG_4 - 0.7-4.2% (Shakib et al., 1975; French, 1986). The wide range of IgG subclass distribution may be due to differences in the assay procedures, and in the populations studied.

Several authors reported differential expression of IgG subclasses in response to various antigens. T-cell dependent responses to viral and bacterial proteins were mainly restricted to IgG_1 and IgG_3 subclasses (Sarnesto et al., 1985; Morrel et al., 1983). The IgG response to carbohydrate antigens including dextran, teichoic acid (Yount et al., 1968) group A streptococcal polysaccharide (Siber et al., 1980; Hammerstrom and Smith, 1983) is restricted to IgG_2 subclass. IgG_4 is associated with atopic allergy (Stanworth, 1983). Chronic antigenic stimulation with common allergens such as grass pollen, house dust, and bee venom causes significant increase in IgG_4 (Alberse et al., 1983; Urbanek et al., 1986). High IgG_4 titres were reported to in parasitic infections (Iskander et al., 1981; Ottesen et al., 1985).

Deficiency of IgG is generally associated with increased risk of infections. In most patients with IgG subclass deficiencies, more than one subclass may be affected. A lack of IgG_1 is usually associated with hypogammaglobulinemia. Other IgG subclass deficiencies may not indicate a reduction in total IgG below normal level.

The rationale for measuring each IgG subclasses in serum is based on the belief that identification of IgG subclass deficiencies that often are marked by normal total IgG can be clinically useful. The production of murine monoclonal antibodies specific for unique determinants on the four human IgG subclasses ushered a new era in the detection and quantitation of IgG subclasses in the clinical immunology laboratory (Hamilton, 1987).

Human immune system has the ability to mount specific immune response against tomour antigens. Since there is a strong association between *H. pylori* infection and gastric cancer development, there could be some immunological changes in patients who have *H. pylori* induced gastric cancer. Many seroepidemiological studies showed increased prevalence of total anti-*H. pylori* IgG in gastric cancer patient sera (Asaka et al., 1994). No detailed reports were available on the levels of human IgG subclasses in gastric cancer patients positive for *H. pylori* infection.

RESULTS

Serum samples from 79 gastric cancer patients of the age between 24-75 were collected for the analysis of anti-*H. pylori* IgG and IgG subclasses. These patients were grouped into five age groups such as group I: 21-40, group II: 41-50, group III: 51-60 group IV: 61-70 and group V: 71-80. The age matched control group consisted pooled serum of 50 healthy individuals. These control serum samples were negative for anti-*H. pylori* IgG.

Table 5.1 shows the levels of anti-*H. pylori* IgG in sera of gastric cancer patients. 72.15% of gastric cancer patients were positive for anti-*H. pylori* IgG. This includes 75.4% men (46/61) and 61.11% of women (11/18).

The analysis of anti-*H. pylori* IgG in the sera of gastric cancer patients of different age groups showed that 80% of the serum samples in the age-group I, 66.6% in group II, 62.5% in group III, 69.56% in group IV and 69.56 % in group V were anti-*H. pylori* IgG positive. The levels of anti-*H. pylori* IgG were at the range of 55-65 EU/ml (as per the experimental protocol) among these age groups.

The standard graphs plotted for each IgG subclass were given in Figures 5.1 to 5.4. The concentrations of each subclasses in serum samples were expressed in mg/ml.

The total IgG (IgG_1+IgG_2+IgG_3+IgG_4) in the control sera was 7.312 mg/ml. The analysis of total IgG in patient sera revealed no significant variation in comparison to control sera. The maximum level of IgG was observed in patients of age group IV (8.0435 mg/ml) (Table 5.2). This increase was observed in both anti-*H. pylori* IgG positive and negative patients of age group IV (8.0863 mg/ml and 7.9457 mg/ml respectively) (Table 5.3 and 5.4). In contrast, although not significant, a decreased level of total IgG was observed in age group I and age group V as compared to the control. This decrease in the total IgG pattern was noted in the sera of gastric cancer patients of the age group V irrespective of anti-*H. pylori* IgG antibody positivity or negativity

The normal control sera contained 4.414±0.2488 mg/ml IgG_1, 2.5013±0.3566 mg/ml IgG_2, 0.3754±0.1803 mg/ml IgG_3, and 0.0213±0.0117 mg/ml IgG_4.

All age groups showed decreased IgG_1 levels when compared to normal control sera. It was observed that there was significant decrease of IgG_1 in all age groups, except group V ($P<0.05$). Maximum decrease in IgG_1 level was observed in age group I (2.745±1.4793 mg/ml) when compared to the control (Table 5.2). In contrast to this an increase in the IgG_2 levels were observed in the sera of gastric cancer patients (Figure 5.5). Since a reverse pattern of IgG_1 and IgG_2 levels was observed in gastric cancer patients, it was of great interest to check IgG subclasses in anti-*H. pylori* IgG positive and negative sera (Table 5.3 & 5.4).

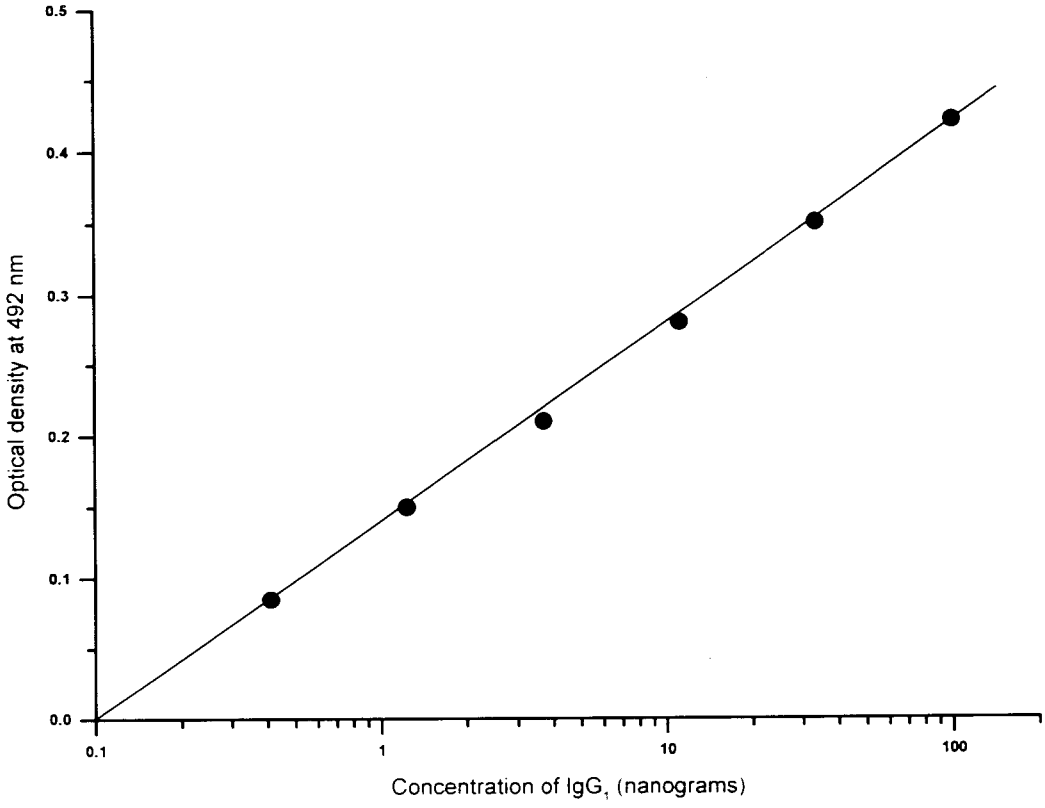

Figure 5.1 Standard curve of IgG_1 (Semi-log graph)

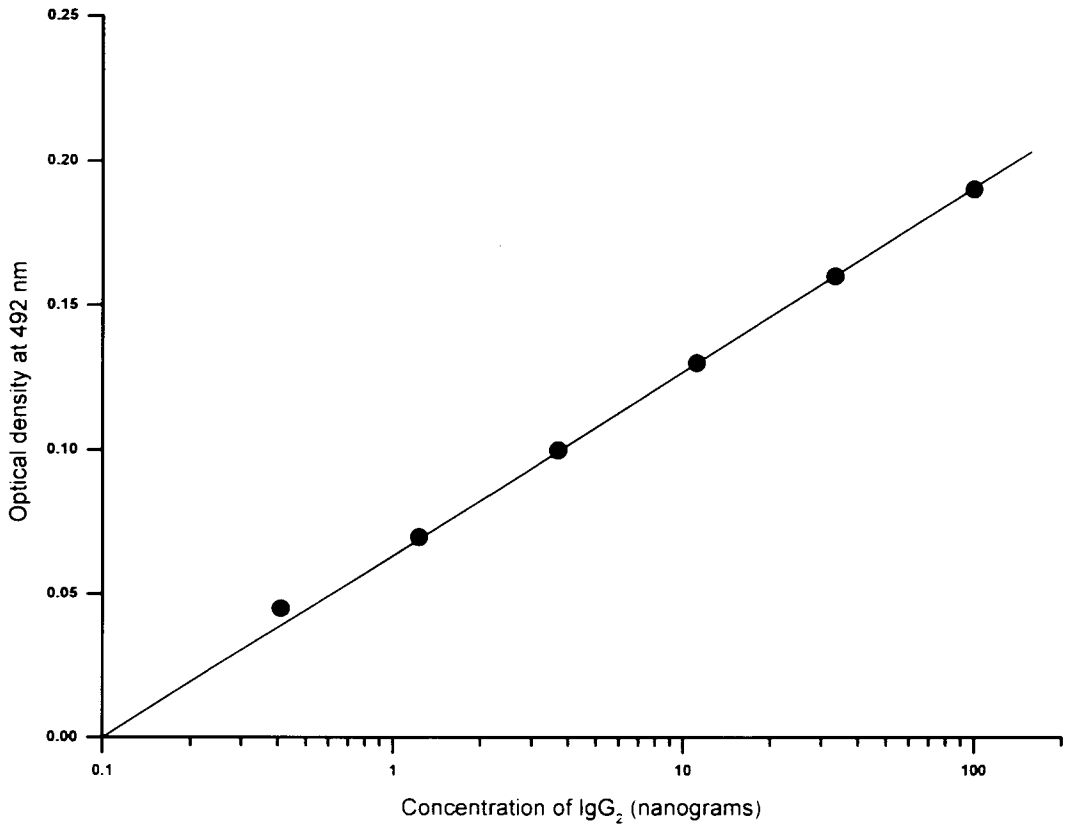

Figure 5.2 Standard curve of IgG_2 (Semi-log graph)

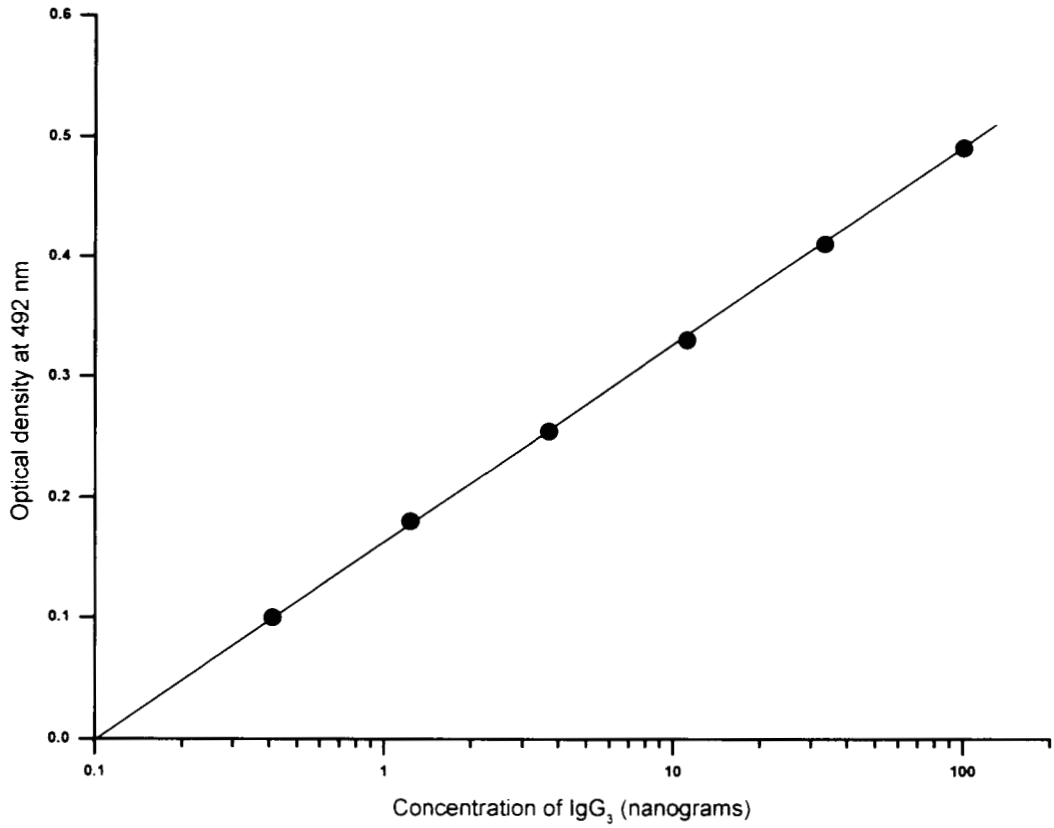

Figure 5.3 Standard curve of IgG_3 (Semi-log graph)

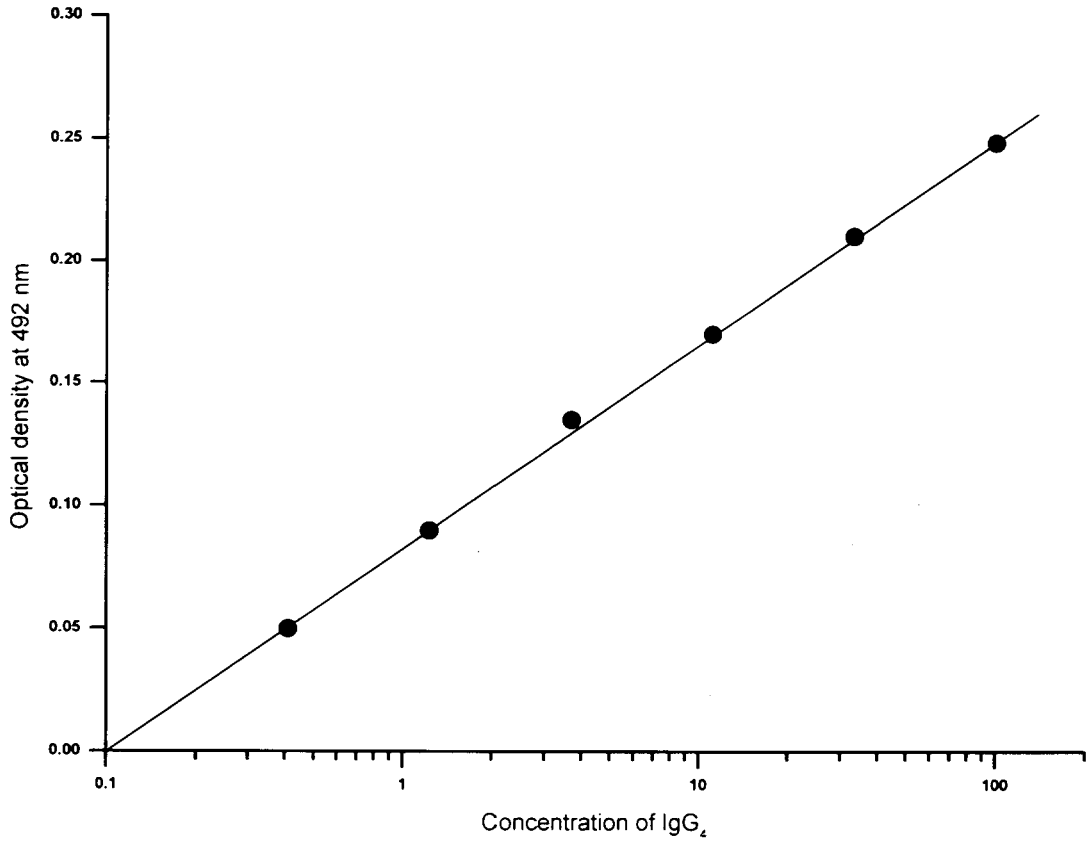

Figure 5.4 Standard curve of IgG_4 (Semi-log graph)

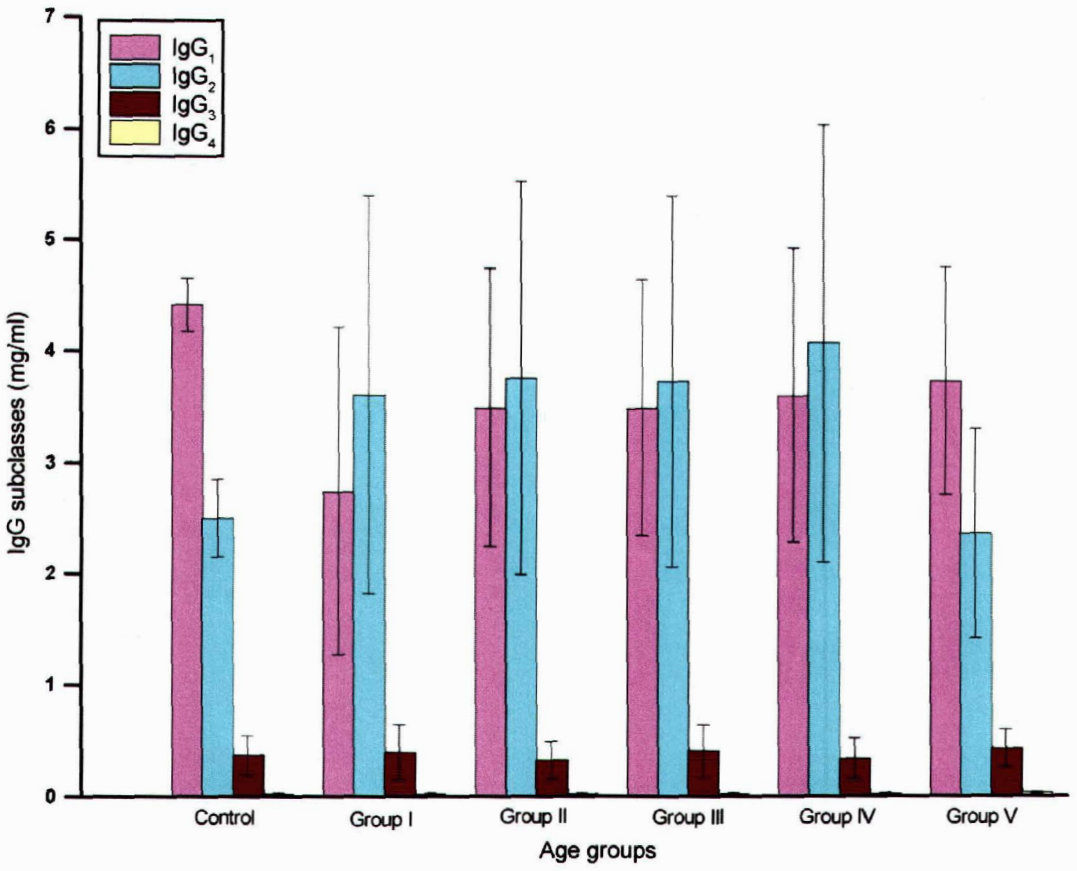

Figure 5.5 Levels of IgG subclasses in gastric cancer patients

The same pattern of decrease in IgG_1 was observed in anti-*H. pylori* IgG positive sera and negative sera when compared to control sera (Figure 5.6 & 5.7). All age groups, except group V, showed significant decrease in IgG_1 level ($P<0.05$) in anti-*H. pylori* IgG positive gastric cancer patients. Among the *H. pylori* negative gastric cancer patients the significant decrease was observed only in age group III (3.2144±0.9824 mg/ml; $P<0.05$).

The analysis of gastric cancer patient sera showed an increase in IgG_2 level, except in group V. The increase in the IgG_2 level was significant in groups II, III, and IV ($P<0.05$).

A significant increase in the level of IgG_2 ($P<0.05$) in anti-*H. pylori* IgG positive samples was noted in the age groups III and IV. In contrast, significantly increased IgG_2 level was noted only in age group IV of the anti-*H. pylori* IgG negative gastric cancer patient sera.

No definite pattern of increase/decrease in IgG_3 levels in gastric cancer patients was observed. IgG_4 levels were relatively remain unchanged in the patients when compared with the controls.

The percentage of each subclass in the normal control and patient sera was calculated. It was found that normal sera contain 60.366% IgG_1, 34.20% IgG_2, 5.13% IgG_3, and 0.292% IgG_4. In the case of gastric cancer patients pattern of decrease in the percentage of IgG_1 in all age groups was observed and this was maximum in group I (Figure 5.8).

On the contrary, a reverse pattern in the percentage of IgG_2 was observed in all age groups, except in group V, of the gastric cancer patients (Figure 5.9).

When G_1/G_2 ratio in the patient sera was analyzed, it was noted that the younger age groups showed maximum decrease and when age increases, the ratio tends to be increased (Figure 5.10). This pattern was same when anti-*H. pylori* IgG positive and negative sera were analyzed separately.

Table 5.1 Levels of anti-*H. pylori* IgG in gastric cancer patients

Age-Groups	Anti-*H. pylori* IgG EU/ml
Group I	55.745±14.740
Group II	65.757±23.843
Group III	60.952±14.581
Group IV	55.572±16.073
Group V	62.178±19.623

Table 5.2 IgG subclasses in gastric cancer patients sera

Age Groups	IgG1 (mg/ml)	IgG2 (mg/ml)	IgG3 (mg/ml)	IgG4 (mg/ml)	Total IgG (mg/ml)
Group I (n=10)	2.7450± 1.4793	3.616± 1.7915	0.4020± 0.2568	0.0248± 0.0193	6.7878
Group II (n=12)	3.4933± 1.2577	3.7698± 1.7731	0.3362± 0.1791	0.0248± 0.0172	7.6250
Group III (n=24)	3.4983± 1.1576	3.7300± 1.6747	0.4118± 0.2451	0.0264± 0.0147	7.6665
Group IV (n=23)	3.6000± 1.3294	4.0750± 1.9788	0.3456± 0.1863	0.0229± 0.0167	8.0435
Group V (n=10)	3.7340± 1.0278	2.3614± 0.9547	0.4308± 0.1774	0.0309± 0.0151	6.5571
Control (n=50)	4.4140± 0.2488	2.5013± 0.3566	0.3754± 0.1803	0.0213± 0.0117	7.3120

Table 5.3 IgG subclasses in anti-H. *pylori* IgG positive gastric cancer patient sera

Age groups	IgG1 (mg/ml)	IgG2 (mg/ml)	IgG3 (mg/ml)	IgG4 (mg/ml)	Total IgG (mg/ml)
Group I (n=8)	2.5762± 1.6702	3.3612± 1.8499	0.4325± 02827	0.0279± 0.0218	6.4025
Group II (n=8)	3.3500± 1.3478	3.6686± 1.7668	0.3773± 0.1774	0.0287± 0.0182	7.4247
Group III (n=15)	3.6686± 1.2518	3.7266± 1.6811	0.4256± 0.2446	0.0288± 0.0154	7.8496
Group IV (n=16)	3.5400± 1.2480	4.1530± 2.2697	0.3663± 0.2098	0.0270± 0.0184	8.0863
Group V (n=7)	3.6542± 0.9735	2.2605± 0.8958	0.4125± 0.1994	0.0334± 0.0163	6.3606
Control (n=50)	4.4140± 0.2488	2.5013± 0.3566	0.3754± 0.1803	0.0213± 0.0117	7.3120

Table 5.4 IgG subclasses in anti-*H. pylori* IgG negative gastric cancer patient sera

Age groups	IgG1 (mg/ml)	IgG2 (mg/ml)	IgG3 (mg/ml)	IgG4 (mg/ml)	Total IgG (mg/ml)
Group I (n=2)	3.420± 1.03	4.635± 2.354	0.261± 0.224	0.0124± 0.0062	8.3280
Group II (n=4)	3.765± 1.208	3.291± 1.336	0.3133± 0.144	0.0294± 0.0152	7.3987
Group III (n=9)	3.2144± 0.9824	3.7355± 1.7657	0.5249± 0.2291	0.0223± 0.0132	7.4971
Group IV (n=7)	3.7370± 1.5982	3.8971± 1.1938	0.2982± 0.1159	0.01348± 0.0056	7.9457
Group V (n=3)	3.9200± 1.3487	2.5966± 1.2550	0.4733± 0.1360	0.0252± 0.0123	7.0151
Control (n=50)	4.414± 0.2488	2.5013± 0.3566	0.3754± 0.1803	0.0213± 0.0117	7.3120

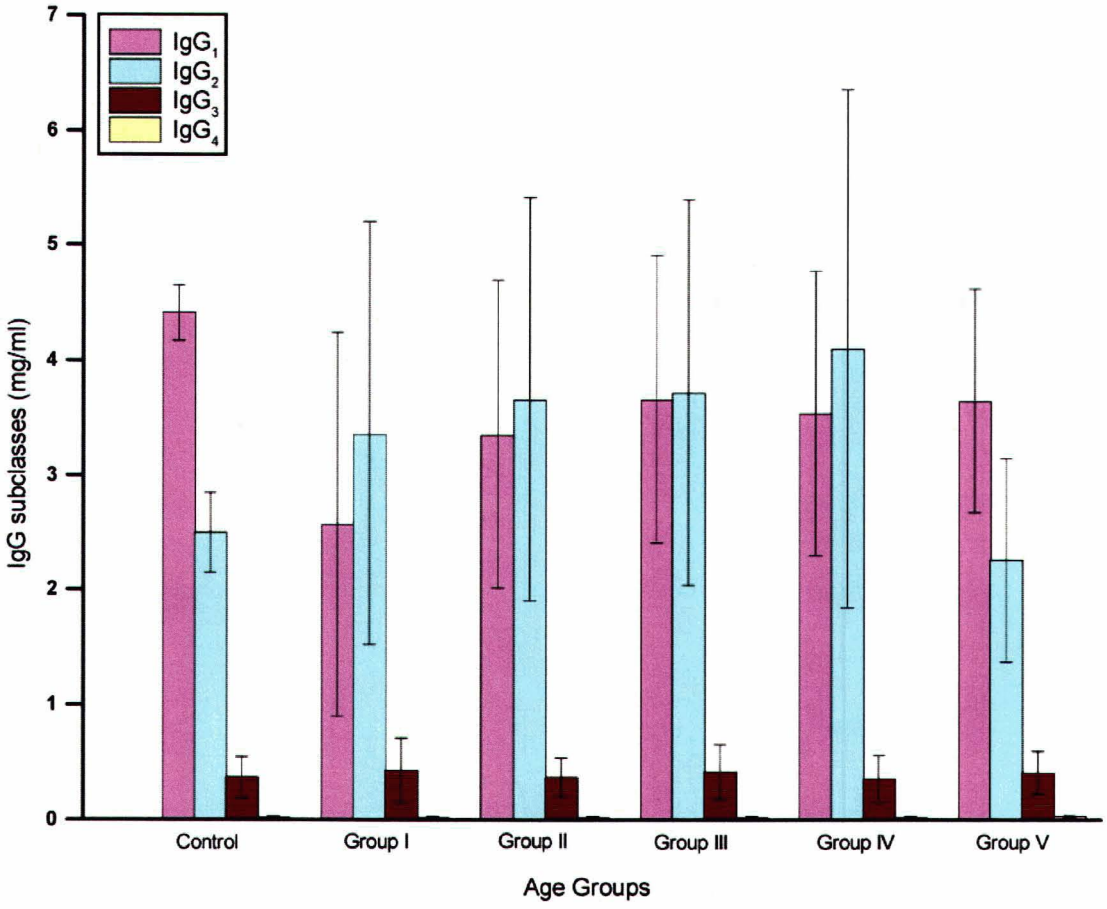

Figure 5.6 Levels of IgG subclasses in anti-*H. pylori* IgG positive gastric cancer patients

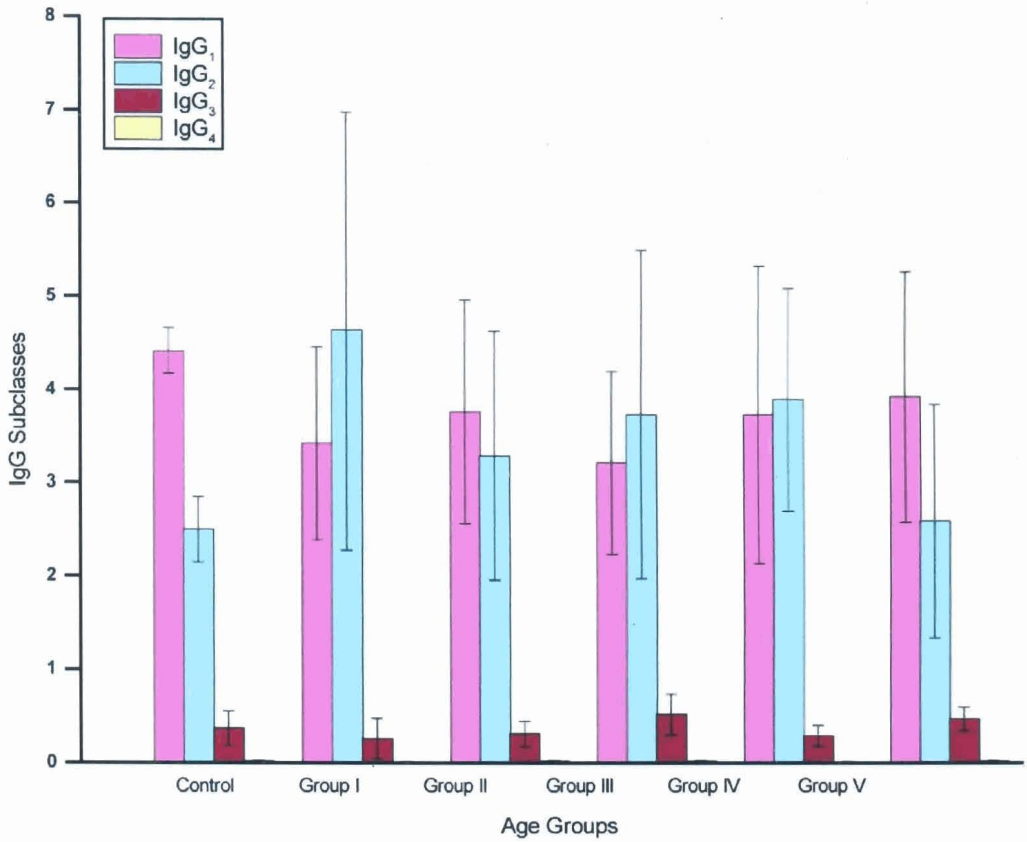

Figure 5.7 The levels of IgG subclasses in anti-*H. pylori* IgG negative gastric cancer patients

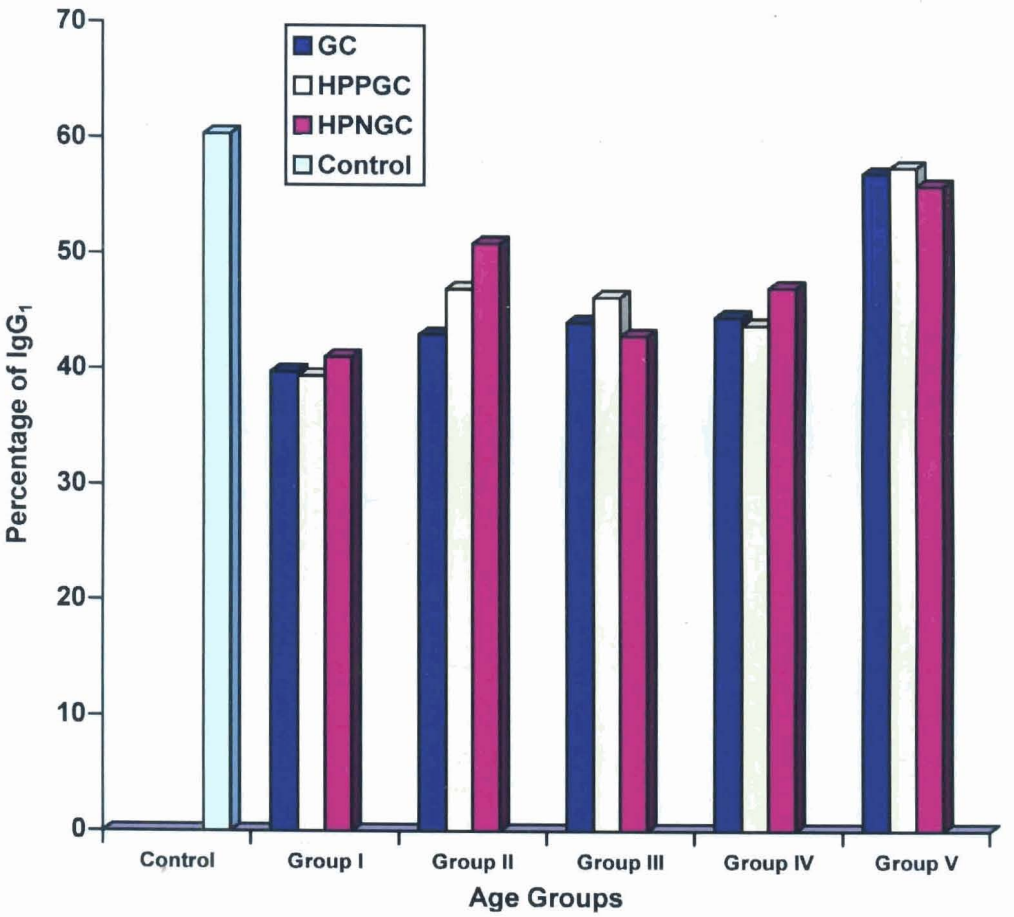

Figure 5.8 Percentage of IgG_1 in patient sera (GC – Gastric Cancer; HPPGC – anti-*H. pylori* IgG Positive Gastric Cancer; HPNGC – anti-*H. pylori* IgG Negative Gastric Cancer)

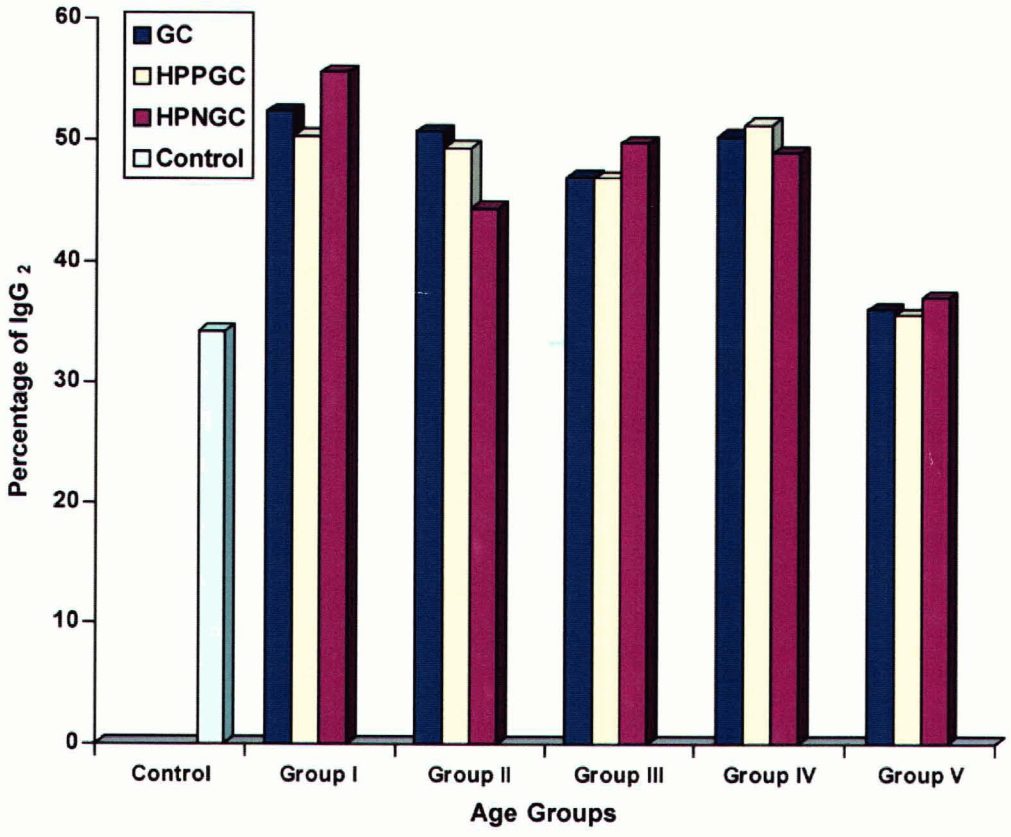

Figure 5.9 Percentage of IgG_2 in patient sera (GC – Gastric Cancer; HPPGC – anti-*H. pylori* IgG Positive Gastric Cancer; HPNGC – anti-*H. pylori* IgG Negative Gastric Cancer)

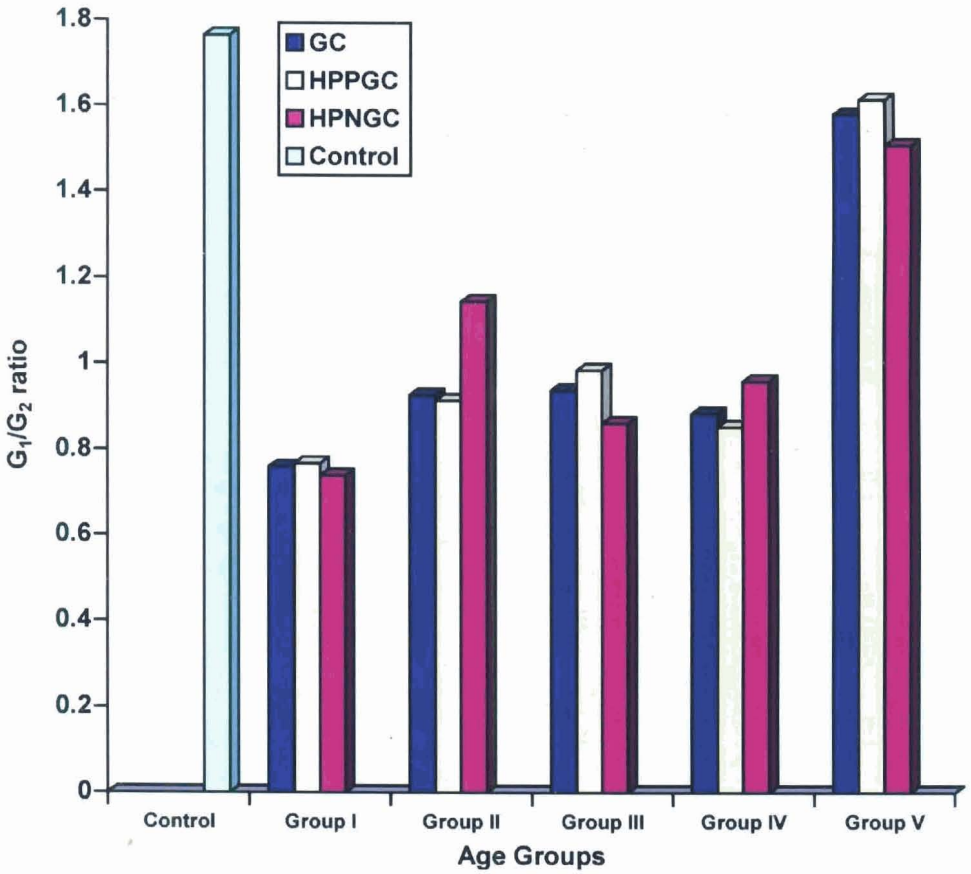

Figure 5.10 G_1/G_2 ratio in patient sera (GC – Gastric Cancer; HPPGC – anti-*H. pylori* IgG Positive Gastric Cancer; HPNGC – anti-*H. pylori* IgG Negative Gastric Cancer)

DISCUSSION

Anti-*H. pylori* IgG in serum samples were detected by using commercially available enzyme immunoassay kits supplied by UBI Magiwel™ . The results were interpreted as negative (less than 30 EU/ml), equivocal (30-40 EU/ml), and positive (greater than 40 EU/ml). Publications are also available in the literature based on the above mentioned kits (Khanna et al., 2002).

72.15% of gastric cancer patient sera were positive for anti-*H. pylori* IgG. The male patients showed higher percentage of seropositivity (75.4%) when compared to female patients (61.11%). Several studies from different countries reported variable results regarding the anti-*H. pylori* IgG prevalence. Case-control studies from high-risk countries such as Japan (Igarashi et al., 1992), Portugal (Estevans et al., 1993), Italy (Miglio et al., 1992), and Taiwan (Lin et al., 1994) showed 73%, 70%, 53% and 62.2% anti-*H. pylori* IgG prevalence in gastric cancer patients respectively. Asaka et al. (1994) reported 88.2% prevalence of anti-*H. pylori* IgG in Japanese gastric cancer patients. A study in India on gastric cancer patients showed 68% seropositivity for *H. pylori* infection (Khanna et al., 2002).

When anti-*H. pylori* IgG was analyzed among different age groups of gastric cancer patients, the maximum prevalence was observed among younger patients (Group I - 80%). Other groups (II, III, IV and V) showed 66.6%, 62.5%, 69.56% and 69.56% respectively. This observation is in agreement with the findings of Kikuchi et al. (2000). Their study showed higher prevalence of anti-*H. pylori* IgG among younger patients. Klaamas et al. (1996) reported 100% *H. pylori* seropositivity in youngest group of gastric cancer patients and Khanna et al. (2002) reported 75% prevalence of anti-*H. pylori* antibodies in gastric cancer patients below the age of 45 and 63% in older patients. The present study did not show any significant difference in the level of anti-*H. pylori* IgG among different age groups of patients.

The level of total IgG (IgG_1+IgG_2+IgG_3+IgG_4) in control sera (7.312 mg/ml) was similar to the data available in the literature (Papadea and Check, 1987). There is no major change in the total IgG level in the different age groups of gastric cancer patients. This observation is in agreement with the earlier observations from this laboratory (Anilkumar, 1993). The level of total IgG was similar in anti-*H. pylori* IgG positive and negative sera

The results showed a general pattern of decrease in IgG_1 and an increase in IgG_2 in gastric cancer patients when compared to controls. The decrease in IgG_1 was significant in all age groups, except for group V ($P<0.05$) and this was similar when anti-*H. pylori* IgG positive samples and negative samples were analyzed separately.

In contrast to the level of IgG_1, an increased level of IgG_2 was observed in gastric cancer patients, except in age group V, when compared to the normal control. The increase in IgG_2 was similar in anti-*H. pylori* IgG positive group and anti-*H. pylori* IgG negative groups.

Among total immunoglobulin producing cells in normal gastric body and antrum. 14% and 12% were IgG producing cells, respectively (Sartor, 2002). Increase in IgG_2 production was reported in diseases like Crohn's disease (Mac Dermott et al., 1988). and inflammatory bowel disease (Scott et al., 1986). This was due to the increased number of intestinal lymphocytes and plasma cells. This phenomenon of the increased number of lymphocytes and plasma cells may also applicable to the gastric epithelium of the gastric cancer patients.

Symptomatic IgG subclass deficiencies were reported. Selective deficiencies of one or more of the IgG subclasses may increase the susceptibility to pyogenic infections (Schur et al., 1970). The development of serum sickness and the presence of IgG_1 antibody and immune complexes were closely related (Bielory et al., 1990).

Children who were evident for IgG_2 deficiency was more prone to infections like sinusitis, pneumonia and bacteriaemia (Shackelford et al., 1986). Stanley et al. (1984)

reported decreased serum IgG_2 concentrations in adults with recurrent acute chest infections or chronic purulent sputum production.

IgG_2 deficiencies alone or in combination with IgG_4 or IgA were reported (Oxelius et al., 1981). In ataxia-telangiectasia patients IgG_2 deficiency was recorded (Oxelius et al., 1982).

Granoff et al. (1988) studied *Haemophilus influenzae* b vaccine failures in children and the results showed an IgG_2 deficiency. The IgG_2 response was found to be sluggish in children immunized with tetanus toxoid and diphtheria toxoid and the IgG_1 response was vigorous (Stevens et al., 1983).

The mean IgG_4 subclass levels were elevated in patients with cystic fibrosis (Shakib et al., 1976), atopic dermatitis (Shakib et al., 1977), and asthma (Gwynn et al., 1978).

In patients with rheumatoid arthritis and in autoimmune thyroiditis, anti-IgG_1 and anti-IgG_4 antibodies were predominant. Some patients with lupus erythematosus showed increased levels of IgG_1 and IgG_3. Thus IgG subclasses may be associated with pathological sequelae of chronic inflammatory diseases (Papadea and Check, 1987). In leprosy patients IgG_2 antibodies were elevated across the disease spectrum and IgG_3 levels were higher in lepromatous than in tuberculoid patients (Buria et al., 1998).

The percentage of IgG_1 decreased in all age groups, while the percentage of IgG_2 increased in the patients. This also showed no relation to *H. pylori* seropositivity. The G_1/G_2 ratio was decreased in younger age groups and as the age increases an increasing tendency of G_1/G_2 ratio observed (Figure 5.10).

The increased production of IgG_2 occurs in human body in response to bacterial polysaccharide antigens (Siber et al., 1980). Hammarstrom et al. (1984) confirmed

predominance of antibodies to Staphylococcal teichoic acid in the IgG_2 subclass in normal subjects, and absent or low titres in IgG_2 deficient subjects.

H. pylori infection can induce strong humoral response systemically and locally (Mattson et al., 1998). Although the data presented here did not show any significant variation in total IgG among different groups of patients when compared to controls, a preferential expression/secretion of IgG_2 subclass was noted. Moreover, a decrease in IgG_1 level with concomitant increase in IgG_2 was noted. Therefore, both patients and control sera exhibited more or less similar levels of IgG.

Fangrat et al. (2003) reported IgG_1 and IgG_3 predominant response stimulated by *H. pylori* in peptic ulcer patients. But the *in vitro* studies with different antigen preparations of clinical isolates of *H. pylori* showed reduction in the induction of IgG_1 with most of the protein antigens and increased IgG_2 production with phenol-water extracts (See chapter IV). Even though the level of IgG_1 is decreased and level of IgG_2 increased in gastric cancer patients, the present study could not differentiate *H. pylori* positive gastric cancer from *H. pylori* negative gastric cancer on the basis of IgG subclasses. The data presented here is based on the analysis of total IgG subclasses. but not IgG subclasses specific to *H. pylori*. This could be the reason for the lack of differentiation.

Several conclusive evidences were established regarding the causative role of *H. pylori* in gastric carcinogenesis. There are reports showing the deficiency of DNA mismatch repair induced by *H. pylori* (Kim et al., 2002). This may increase the risk of mutation accumulation in gastric mucosal cells and risk of gastric cancer development during chronic *H. pylori* infection. Genetic alterations occur in oncogenes, tumour suppressor genes, cell adhesion molecules, and genetic instability. Asaka et al. (2001) summarized the genetic alterations observed in well-differentiated and poorly differentiated gastric carcinomas. Studies in Indian population showed high prevalence

of *H. pylori* infection that leads to premalignant conditions (Kumar et al, 2002; Mukhopadhyay et al., 1992).

The study of Matsui et al. (1995) suggested the inhibition of growth of gastric cells in presence of ammonia and ammonium chloride. Some studies supported the role of cyclin-dependent kinase (cdk) inhibitors in the G1 cell-cycle arrest exerted by *H. pylori* and its involvement in changing the regulatory proteins p35, p21 and cyclin E in the cell cycle (Ahmed et al., 2000).

The prevalence of *H. pylori* infection is reported to be much higher among subjects with a parental history of stomach cancer (Brenner et al., 2000). Increased prevalence of pre-cancerous gastric abnormalities in relatives of patients with gastric cancer was mainly confined to those with *H. pylori* infection. These findings partially explain the familial clustering of gastric cancer.

Final proof for the association of *H. pylori* infection with the gastric cancer development was evidenced from animal model experiments. In Mongolian gerbils. Sugiyama et al. (1998) demonstrated the increase in the development of MNU-induced gastric cancer in presence of *H. pylori*. In the same year, Watanabe et al. (1998) reported the induction of gastric adenocarcinoma in Mongolian gerbils due to long term *H. pylori* infection.

Many immunization studies in mice models revealed the induction of IgG_1 and IgG_2a subclass in response to whole *H. pylori* organism or antigens derived from *H. pylori* (Arcila et al., 2002; Shirai et al., 2000). Coutelier et al. (1988) reported the virally modulated murine IgG subclasses response to IgG_2a instead of IgG_1 and IgG_3 subclasses. These observations indicated that pathogens can actively influence the switch of immunoglobulins and selectively stimulate the production of specific IgG subclasses.

Several reports demonstrated the selection of Th1 pathway of immune response in patients with *H. pylori* infection (Reviewed by Ibrahimov and Papo, 2000). This may be modulating the humoral response against the organism in the human host.

Thus, it is concluded that gastric cancer is multifactorial in origin and *H. pylori* plays a role in the initial step as a causative agent for chronic inflammation. The levels of IgG subclasses could not differentiate *H. pylori* positive and *H. pylori* negative gastric cancers.

Chapter VI

ACTIVATION OF MACROPHAGES

CHAPTER VI

ACTIVATION OF MACROPHAGES

Inflammation is the primary process through which the body repairs tissue damage and defends itself against infection. Both immune pathways and non-immune pathways initiate inflammation. *In vivo* effects of immune activation underlying the inflammation are determined by amplification mechanisms. Rather than the specific immune reaction alone, the amplification mechanisms are largely responsible for the tissue lesions actually observed. As a physiological process to injury, inflammation clears and restores damaged tissue and as a pathological process, inflammation produces tissue damage (lesions).

The cellular components in the process of acute inflammation include mast cells, neutrophils, platelets and eosinophils, while in chronic inflammation lymphocytes, macrophages and plasma cells, collectively termed as mononuclear cells, were playing major role (Sell, 1987). Marshall and Warren (1984) noted the presence of *H. pylori* almost always in polymorphonuclear cell infiltrated mucosa and in the absence of inflammation, the bacteria were rarely observed.

Several studies followed after the announcement of Marshall and Warren proved definite association of *H. pylori* infection with gastric inflammation and development of ulcer. The multiplication of the organism in close proximity to the surface of epithelial cells results in mucin depletion, cellular exfoliation and compensatory regenerative changes. During this acute phase of infection polymorphonuclear cell infiltration into foveolar and surface epithelium, and lamina propria edema are conspicuous. Collections of polymorphs in the foveolae and adherent neutrophil exudates on the surface may also be present (Dixon, 2001).

The major mediators of acute response include bacterial lipopolysaccharide and chemotactic factors (Slomiany et al., 1998; Crabtree, 1994). *H. pylori* stimulate IL-8

production by gastric epithelium, which is up regulated by TNF-α and IL-1 released by activated macrophages in response to lipopolysaccharide (Crabtree, 1994). Soluble *H. pylori* proteins caused additional IL-8 release by polymorphs themselves (Kim et al., 1998). Mast cell activation by *H. pylori* leading to the release of acute inflammatory mediators contributes to the acute phase of infection (Graham, 1992).

Failure to clear the pathogen by acute phase response leads to the accumulation of chronic inflammatory cells into the gastric mucosa. Persistent antigenic stimulation leads to the development of follicles and follicles will always be found in *H. pylori* infected stomachs (Genta et al., 1993). Follicle formation seems to be a universal response to *H. pylori* infection.

The inflammatory cells are activated by exposure to bacterial lipopolysaccharides, peptidoglycan-polysaccharide complexes and proinflammatory cytokines. The relative balance of proinflammatory and protective cytokines and inflammatory mediators determines tissue injury versus protection (Sartor, 2002). Mutually exclusive, well-demarcated Th1 versus Th2 lymphokine profiles characterizes chronic inflammation. Th1 cells are generally considered to be "proinflammatory" in the context of the digestive tract as the production of the IFN-γ and TNF-α contributes to the chronic inflammation including gastritis associated with *H. pylori* infection (Wang et al., 2001). Th2 cells serves to keep Th1 response in check.

Romagnani et al. (1996) detected Th1 dominated response in the gastric antrum of *H. pylori* infected subjects with peptic ulcer. Majority of Th1 cells present in the biopsies showed specificity to *H. pylori* antigens. $CD4^+$ T-cells are both necessary and sufficient for gastritis. IFN-γ contributes to the $CD4^+$ T-cell induced gastritis, while IL-10 suppress it (Eaton et al., 2001). *H. pylori* preferentially induced IL-12 rather than IL-6 or IL-10 in human dendritic cells (Guinea et al., 2003). Helical form of *H. pylori* induced higher amount of IL-8 secretion from cultured MKN-45 cells compared to coccoid forms (Osaki et al., 2002). In response to purified *H. pylori* urease stimulation.

IL-6 and TNF-α predominated rather than IL-8 (Tanahashi et al., 2000). It was assumed that an evolutionarily conserved strategy to disrupt a Th2 response and evasion of the host immune system allows *H. pylori* for successful chronic infection (Ceponis et al., 2003).

$CD4^+$ T-cell dependent *H. pylori* gastritis leads to epithelial damage with attendant proliferative and metplastic response (Peterson et al., 2003). The local TNF-α, IL-6 and IL-8 concentrations in antral biopsy samples of *H. pylori* infected persons were higher than those in *H. pylori* negative persons (Klausz et al., 2003).

Nitric oxide is one of the soluble mediators of inflammation. Nitric oxide synthase (NOS) oxidizes arginine to nitric oxide, which has multiple biologic properties relevant to mucosal inflammation (Kubes and McCafferty, 2000). Inducible nitric oxide synthase (iNOS) is produced in intestinal epithelial cells, macrophages, and mesenchymal cells in response to lipopolysaccharide, IL-1, TNF, IFN-γ and invasive bacteria through the nuclear factor κB (NFκB) signal transduction pathway (Jobin and Sartor, 2000). Patients infected with *H. pylori* expressed more iNOS and higher levels of iNOS were induced by infection with CagA positive strains (Li et al., 2003). Increased levels of iNOS and cyclooxygense-2 were demonstrated in *H. pylori* associated gastritis (Fu et al., 1999).

An *in vitro* study by Watanabe et al. (2000) proved the role of increased iNOS expression in response to *H. pylori* and the authors suggested the role of NO in gastric cell apoptosis and the consequent cell injury. Mizuki et al. (2000) suggested increased mucosal DNA damage as a result of increased neutrophil oxidative burst stimulated by *H. pylori*.

Some studies demonstrated the presence of neutrophil activating protein, which increased adhesion of neutrophils to endothelial cells *in vivo* and *in vitro* in presence of *H. pylori* water extracts (Yoshida et al., 1993). Later this protein (HP-NAP) was isolated and found to be a 150 kD multimeric protein (Evans Jr. et al., 1995). Moreover,

anti-phagocytic activity of *H. pylori* was demonstrated both with murine and human macrophages (Ramarao and Meyer, 2001). This activity was dependent on cagPAI. Thus, *H. pylori* is able to activate the infiltrating phagocytic cells, at the same time it is able to inhibit its own uptake by activated cells. These effects may intensify the pathological events in the infected gastric mucosa.

Keeping in mind the above information, the activation of murine macrophages in presence of different *H. pylori* antigens was carried out.

RESULTS

Among the various concentrations of antigens and mitogens tested, maximum stimulation of macrophage activity was obtained with 10 µg/ml of antigens and 50 µg/ml of mitogens (ConA and PHA). Therefore, these concentrations were taken as optimum concentration. The results of NO generated and lysosomal enzyme activities after the stimulation of murine macrophages with antigens from *H. pylori* isolate strains and mitogens are shown in Figures 6.1 to 6.3.

The enzyme activities and nitric oxide (NO) generated in the cell lysates after the cells were incubated in presence of antigens from *H. pylori* isolate strains and mitogens was compared with the control. In control wells 3.7196 nM NO/10^6 cells/24 h, 1.59 nM p-nitrophenol/10^6 cells/h, and 0.223 nM phenolphthalein/10^6 cells/h were obtained. These values correspond to the NO generated, acid phosphatase activity, and β-glucouronidase activity, respectively, in un-stimulated macrophages *in vitro*.

The amounts of NO generated in presence of different antigens of *H. pylori* isolate strains were compared with control and it was found that the maximum amount of NO was generated in presence of whole cell sonicate antigens. In fact the NO generated by these antigens was higher than the NO generated in presence of PHA and ConA (Figure 6.1).

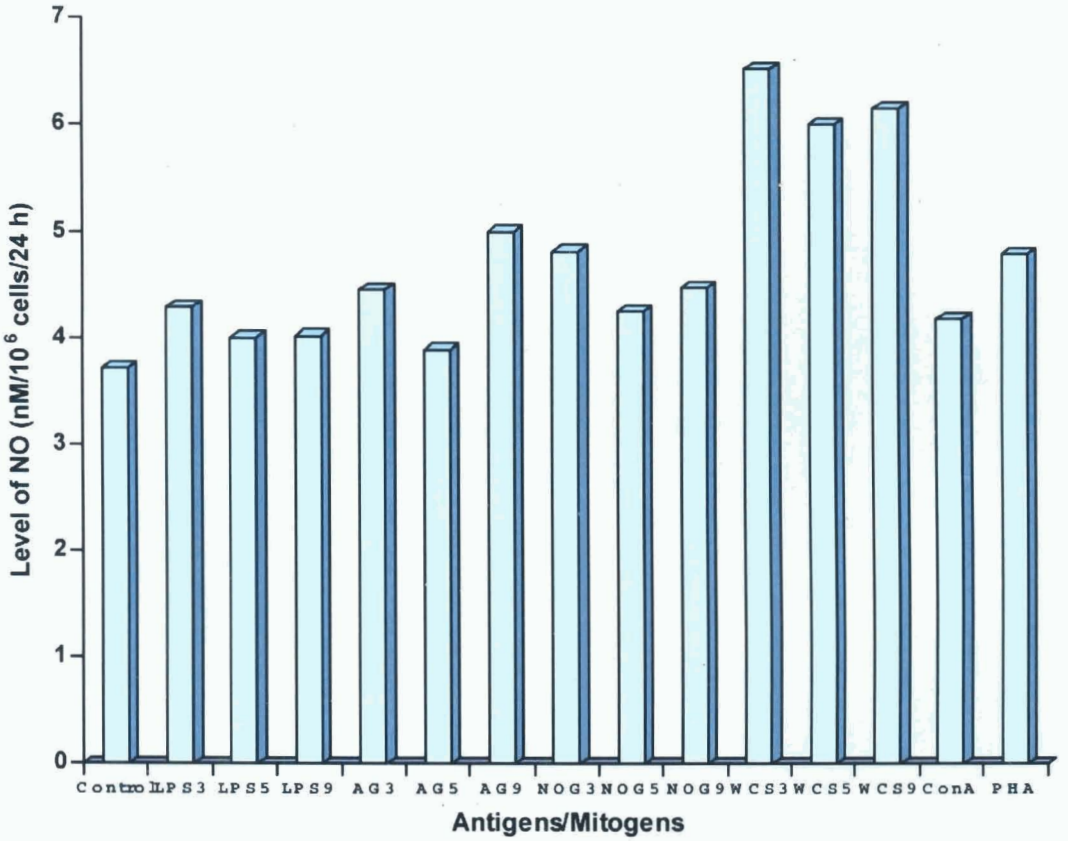

Figure 6.1 Levels of nitric oxide generated in response to the stimulation with *H. pylori* antigens/mitogens [Phenol water extract (LPS), AG (Acid-glycine extract), NOG (n-octyl-β-D-glucopyranoside extract), and WCS (Whole cell sonicate) preparations of strains 3, 5, and 9]

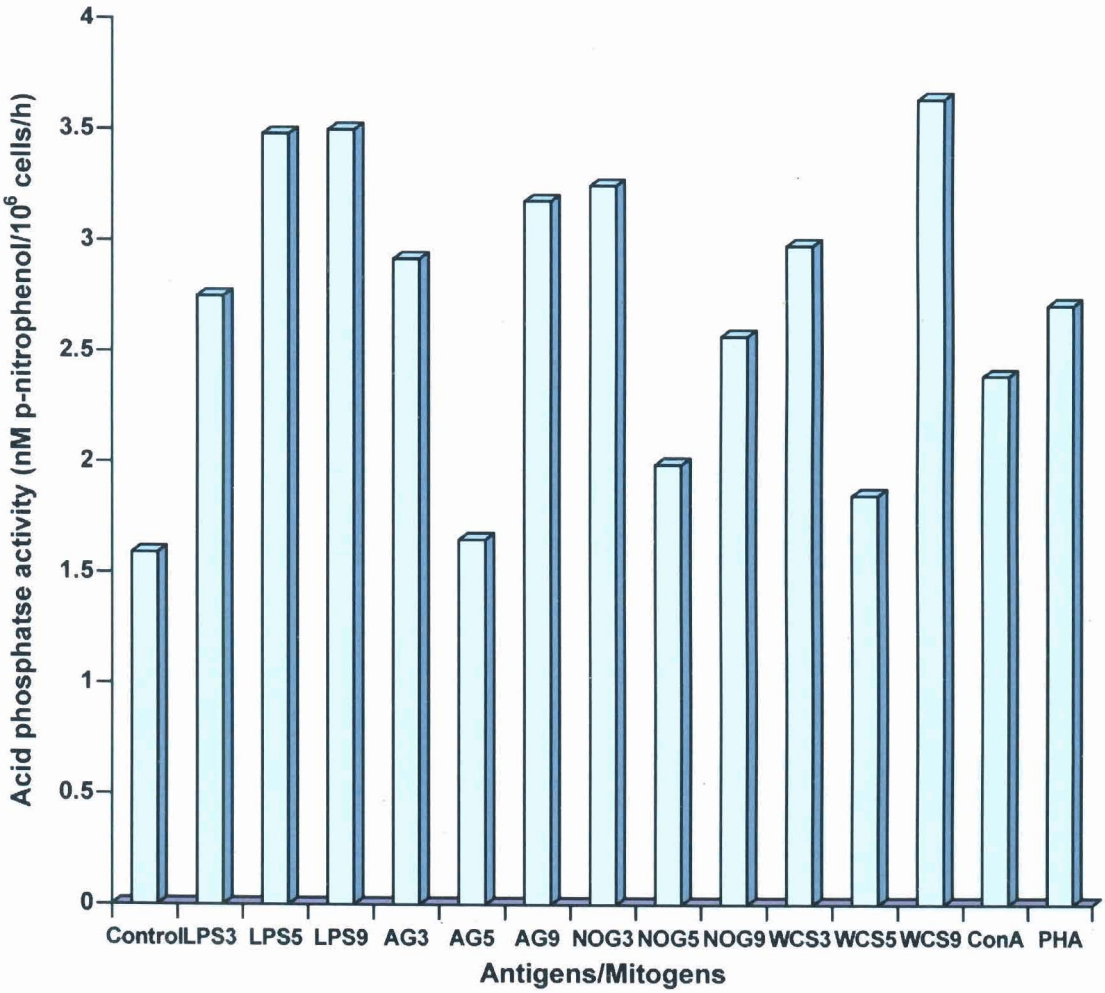

Figure 6.2 Levels of acid phosphatase activity obtained in response to the stimulation with *H. pylori* antigens/mitogens [LPS (Phenol water extract), AG (Acid-glycine extract), NOG (n-octyl-β-D-glucopyranoside extract), and WCS (Whole cell sonicate) preparations of strains 3, 5, and 9]

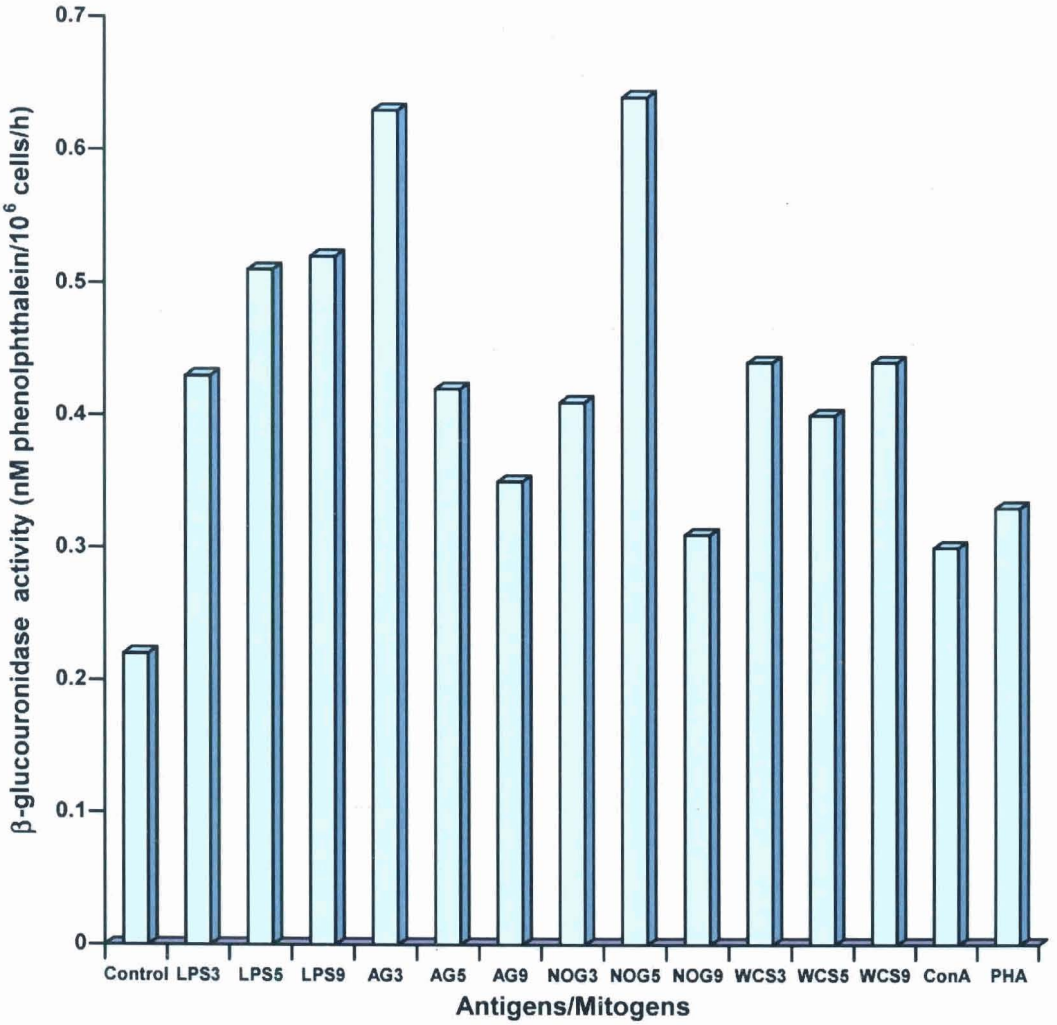

Figure 6.3 Levels of β-glucouronidase activity obtained in response to the stimulation with *H. pylori* antigens/mitogens [LPS (Phenol water extract), AG (Acid-glycine extract), NOG (n-octyl-β-D-glucopyranoside extract), and WCS (Whole cell sonicate) preparations of strains 3, 5, and 9]

The ratio of the enzyme activities and NO generation between the experiment and the control cells was calculated (Table 6.1). The data obtained reveals that there was an increase in NO generation by the macrophages when the cells were incubated with various antigens of *H. pylori* isolates and this was more than the NO generated in presence of known mitogens like ConA and PHA, except in presence of phenol-water extract antigens and acid-glycine extract of strain No. 5.

The level of activities of lysosomal enzymes, acid phosphatase and β-glucouronidase, from the lysates of *in vitro* cultured macrophages were estimated by using specific substrates of the enzymes. The data obtained is presented in the Figure 6.2 and 6.3. It was observed that the level of activities of these enzymes was increased when compared to control. All the antigens from the *H. pylori* isolate strains showed increase in the acid phosphatase activity. When the ratio of activity with control was analysed, it was observed that there was more than 1.5-fold increase in the acid phosphatase activity, except for acid-glycine extract, n-octyl-β-D-glucopyranoside extract and whole cell sonicate antigens of isolate strain 5. In the case of β-glucouronidase activity, more than 1.5-fold increase in the β-glucouronidase activity was observed with the antigens of the *H. pylori*, except n-octyl-β-D-glucopyranoside extract of isolate 9. This increase in activity was higher than that induced by ConA and PHA. The mitogens ConA and PHA induced only 1.37-fold and 1.49-fold increase in β-glucouronidase activity, respectively.

From the above data it can be concluded that the *H. pylori* isolate strains were highly efficient in stimulating macrophages. The concentration of different antigens required for macrophage activation was lower than the concentrations of mitogen. The optimum concentration of the mitogens required to stimulate macrophages was 50 µg/ml as compared to 10 µg/ml of antigen.

Table 6.1 Ratio of the Nitric oxide generation and enzyme activities in comparison with control

Antigens/ Mitogens	NO generation	Acid phosphatase	β-glucouronidase
LPS3	1.155	1.732	1.9349
LPS5	1.0771	2.1926	2.2959
LPS9	1.0771	2.1926	2.2959
AG3	1.1977	1.8368	2.8497
AG5	1.048	1.0396	1.8856
AG9	1.3459	2.0047	1.5874
NOG3	1.2935	2.0486	1.852
NOG5	1.1434	1.2553	2.8781
NOG9	1.2018	1.6173	1.3923
WCS3	1.7513	1.8773	2.0089
WCS5	1.6168	1.1641	1.8161
WCS9	1.6558	2.2943	2.0067
ConA	1.122	1.5031	1.3744
PHA	1.2896	1.7100	1.4955

DISCUSSION

In developing countries, *H. pylori* induced gastric cancer development occurs in only a minority of infected individuals, and the majority remain asymptomatic through out their life (Blaser, 1992; Valle et al., 1996). There are differences in the mucosal injury developed in *H. pylori* infected individuals. This could be probably due to the differences in the virulence between strains, variation in host immunity, situation of acid secretion and differences in the timing of infection (Kuipers; 1998).

H. pylori infection is characterised by infiltration of inflammatory cells into gastric mucosa. *H. pylori* has well-developed mechanism for modulation of immune response towards Th1 phenotype in the mucosa (Ceponis et al., 2003). This Th1/Th2 paradigm of immune response may be useful to explain the inflammatory reactions involved in the pathogenesis of gastrointestinal disorders (Romagnani et al., 1996).

The main function of the inflammation is to deliver plasma and cellular components of the blood to extravascular tissue. The consequent oedema causes dilution of toxic materials; infiltrated phagocytic cells destroy pathogens and other materials.

Inflammatory macrophages were larger, display more ruffling of the plasma membrane and have increased protein content in the cell. They exhibit increased pinocytosis and phagocytosis. In every 15 to 30 min, activated macrophages internalise the equivalent of their entire surface area (Steinman et al., 1983). This marked increase in macrophage endocytic activity is associated with coincident increase in numbers of lysosomes and lysosomal fusion vacuoles. Levels of more than 40 hydrolytic lysosomal enzymes necessary for digestive function rise 5-fold to 20-fold (Meltzer and Nacy, 1989).

For identifying lysosomes various cytochemical techniques are used. Most important among these are cytochemical detection of acid phosphatase, β-glucouronidase, aryl sulphatase, N-acetyl-β-glucosaminidase and 5-bromo-4-chloroindoleacetate-esterase (De Robertis and De Robertis Jr., 1995). In the present study the levels of acid phosphatase and β-glucouronidase activities were analysed to assess lysosomal activity in cultured macrophages in response to antigen stimulation.

The relationship between macrophage cytotoxicity and enhanced production of reactive nitrogen and oxygen intermediates is well established. Nitrite is a stable oxidative end product of the antimicrobial effector nitric oxide produced by phagocytic cells (Green and Nacy, 1993). Reactive nitrogen and oxygen intermediates are two types of effector molecules, which are extensively evaluated in immunologically activated macrophages.

H. pylori infection is associated with chronic inflammation and several authors reported inflammation mediated tissue damage in the pathogenesis of the organism (Marshall and Warren, 1984; Blaser, 1990). Strong activation of neutrophils by strains of *H. pylori* was reported (Rautelin et al., 1993). Ability to stimulate the neutrophil oxidative burst varies among *H. pylori* strains (Mizuki et al., 2000).

The data obtained in the present study shows that all antigens from the *H. pylori* isolate strains stimulate murine macrophages efficiently. When NO generation was analysed, it was seen that the whole cell sonicate antigens induced maximum level of NO when compared to other antigens. This data is in agreement with the results of Noorgaard et al. (1995), who observed increased neutrophil oxidative burst with sonicate antigens of *H. pylori*.

The phenol-water extract antigens showed the least induction of NO. Several workers demonstrated the low biological activities of *H. pylori* lipopolysaccharides (Perez-Perez et al., 1995; Nielsen et al., 1994).

Even though, phenol-water extract antigens have low-level activity, the surface proteins of the organism are efficient in stimulating macrophage activities. Mai et al. (1991) reported the lipopolysaccharide independent mononuclear cell activation by surface proteins of *H. pylori*. The present data agrees with this finding.

The lysosomal enzyme activities were increased in the presence of different antigens. This also indicates activation of murine macrophages in presence of *H. pylori* antigens.

The evidence for the infiltration of inflammatory cells into the gastric mucosa was explained by the presence of various cytokines which are acting as chemokines. It has been proved that the early stages of *H. pylori* infection induces the production of chemokines RANTES, GRO α, MIP 1-α, ENA-78, MCP-1 and IL-8, as well as secretion of the proinflammatory cytokines IL-1, IL-6, and TNF α (Bodger and Crabtree, 1998). Thus infiltration of macrophages, neutrophils, mast cells, and T and B-lymphocytes to the infected gastric tissue occurs. The increased production of IL-8, a potent neutrophil activating chemokine, by epithelial cells in response to *H. pylori* infection have been observed (Osaki et al., 2002). Animal experiments proved the infiltration of neutrophils in gastric mucosa in parallel to the expression of IL-8 due to *H. pylori* infection (Rossi et al., 2000). Purified *H. pylori* urease induced IL-6 production from cultured human epithelial cells (Tanahashi et al., 2000).

One major consequence of the increased phagocytic activity in the infected tissue is the production of reactive nitrogen intermediates. Both lipopolysaccharide and cytokines were shown to stimulate NO production (Leaf et al., 1991). The studies of

Watanabe et al. (2000) indicated that inducible nitric oxide synthase (iNOS) expression plays an important role in gastric cell injury.

NO is known to decompose rapidly in the presence of O_2 to yield a variety of nitrogen oxides and potent N-nitrosating agents capable of forming endogenous carcinogenic N-nitrosocompounds. Additionally, NO may react with superoxide radicals to form the peroxynitrite ion, which may decompose further to form the highly mutagenic hydroxyl radical (Esumi and Tannenbaum, 1994). The hydroxyl radicals were important in gastric cancer development (Salim, 1994). Some reports showed enhanced nitric oxide synthase (NOS) gene expression in macrophage lines in response to soluble proteins from *H. pylori* (Chi et al., 1994). Increased neutrophil oxidative burst stimulated by *H. pylori* may play a role in enhanced gastric mucosal DNA damage (Mizuki et al., 2000). Thus, the infection may contribute to the development of gastric cancer.

Ramarao et al. (2000) observed the ability of *H. pylori* to inhibit its own uptake by freshly isolated neutrophils and monocytes. Later studies proved that *H. pylori* not only inhibits its own phagocytosis, but in trans it can inhibit the phagocytosis of co-infecting *Neisseria gonorrhoeae* by human and murine macrophages (Ramarao and Meyer, 2001). This antiphagocytic activity of the organism was depended on the cag pathogenicity island (cagPAI). Thus the organism is able to activate the infiltrated cells. at the same time it can resist phagocytosis. Consequently, the degranulation and the release of proteolytic enzymes may occur in the infected tissues. It is believed that degranulation of neutrophils and the resultant release of proteolytic enzymes could be contributing factor in the tissue damage and this is evident in other chronic inflammatory disorders (Wendling et al., 1991). The leakage or secretion of proteolytic enzymes outside of the phagocytic cells occurs either during phagocytosis or after exposure of the phagocytes to the antibody-coated surface or a soluble stimulus.

Outside the cell, within the tissues, they can attack extracellular targets (Klebanoff, 1998).

In the present study, increased levels of acid phosphatase and β-glucouronidase activities were obtained in presence of various antigens of *H. pylori* isolate strains. This indicates the increased number of lysosomes within the macrophages. Thus, the possibilities of tissue damage mediated by lysosomal enzymes in *H. pylori* infected tissues were anticipated.

When all parameters were taken together, it is found that *H. pylori* antigens were highly efficient in inducing macrophage activation. Especially low molecular weight soluble proteins are showing high efficiency when compared to LPS preparations. So it is concluded that the inflammatory response and consequent events such as secretion of lysosomal enzymes and increased production of NO due to *H. pylori* infection may contribute to the pathogenesis of infection.

Chapter VII

ANALYSIS OF LIPID PROFILE IN GASTRIC CANCER PATIENT SERA

CHAPTER VII

ANALYSIS OF LIPID PROFILE IN GASTRIC CANCER PATIENT SERA

Several biochemical changes were reported to be associated with *H. pylori* infection. In persons with active *H. pylori* infection an increased level of fasting and meal-stimulated serum gastrin was observed (Graham et al., 1990; Levi et al., 1989). Elevated levels of serum pepsinogen were correlated with *H. pylori* infection (Asaka et al., 1992; Mertz et al., 2000). *H. pylori* eradication therapy caused reduction in the levels of serum gastrin and pepsinogens (Al-Assi et al., 1999). The level of anti-oxidants, such as vitamin C, was found to be lower in *H. pylori* infected persons and eradication therapy improved the level of antioxidants (Rokkas et al., 1995). But the level of ghrelin, a peptide produced mostly in the stomach of rodents and humans and is secreted into the blood stream, does not influence by *H. pylori* infection (Gokeel et al., 2003).

The studies in acute myocardial infarction in humans revealed the association of infectious diseases as risk factors. Pasceri et al. (1998) reported association of virulent *H. pylori* strains with ischaemic heart disease. Several recent reports confirmed this association. Anti-*H. pylori* IgA antibody positivity was higher in acute myocardial infarction patients (Hara et al., 2001). The CagA positive strains were reported to be highly associated with atherosclerotic stroke (Pietrisusti, et al., 2002).

The cytotoxin producing strains of *H. pylori* might enhance the atherosclerotic process by inducing a persistent, low-grade inflammatory response in arterial wall with enhanced synthesis of acute phase reactants (Pieniazek et al., 1999).

Increased cholesterol is a causative factor in the aetiology of atherosclerotic disease. The accumulation of free and esterified cholesterol in the aorta, coronary arteries, and cerebral vessels were the cause of atherosclerotic stroke and the rate of accumulation these lipid components varies among individuals. The incidence and prevalence of coronary heart disease (CHD) are high when total cholesterol concentration is high (Gordon et al., 1981). The risk of coronary heart disease increases as the cholesterol level increases, and at concentrations of 200-240 mg/dL,

the risk begins to accelerate at a greater magnitude. On average, each 1% reduction in cholesterol (2-3 mg/dL) results in about 2% reduction in coronary heart disease incidence (Lipid research clinic coronary disease primary prevention trial result I & III).

A modified lipid profile was reported in *H. pylori* infected subjects (Chimienti et al., 2003). Increased levels of cholesterol, LDL-cholesterol and cholesterol-HDL-cholesterol atherogenic index was observed in *H. pylori* positive non-cancerous patients. Significant differences in the level of Lipoprotein (a) were observed between infected and uninfected subjects. Earlier, Laurila et al. (1999) reported significantly increased levels of triglycerides and total cholesterol in male patients who were positive for IgG and IgA antibodies to *H. pylori* than in the males with no signs of infection.

The above reports supports the findings in seroepidemiological studies showing significant association between *H. pylori* infection and coronary heart disease. Evaluation of the anti-*H. pylori* IgG and anti-CagA IgG antibodies showed increased risk of coronary heart disease in *H. pylori* positive patients (Pieniazek et al., 1999). Zito et al. (1999) reported increased levels of fibrinogen in *H. pylori* infected myocardial infarction patients and controls. This may be the possible mechanism by which *H. pylori* increases myocardial infarction and the concomitant conditions, like genetic predisposition in increasing fibrinogen levels, seem to further increase the effect of *H. pylori* on myocardial infarction risk.

H. pylori have been considered as group 1 (definite) carcinogen by IARC, and several experimental models and seroepidemiological studies gave evidences for the causative role of the organism in gastric cancer development. The lipid profile analysis of gastric cancer patient sera showed hypocholesterolemia (Anilkumar, 1993), which was consistent with the results, reported elsewhere (Alexopoulose et al., 1987). Many authors considered *H. pylori* infection as the risk factor for the development of acute myocardial infarction (Hara et al., 2001; Pellicano et al., 2002). There were no reports available on the analysis of lipid profile in gastric cancer patients in relation to *H. pylori* infection. Therefore, the analysis of lipid components in the serum of gastric cancer patients in relation to *H. pylori* infection was of great interest of the present study.

RESULTS

The results of lipid profile analysis in the sera of gastric cancer patients are shown in Table 7.1. The patients were grouped into five age groups as in the case of IgG subclass analysis (Chapter V). 72.15% of the patient serum samples were positive for anti- *H. pylori* IgG.

The levels of total cholesterol, HDL-cholesterol and LDL-cholesterol were more or less same or lower in gastric cancer patient sera than the control sera (Table 7.1). However, the levels of triglycerides and VLDL-cholesterol were higher in all age groups when compared to the control.

A significant reduction in the total cholesterol and LDL-cholesterol was noted in all age groups ($P<0.05$) (Figure 7.1), except age group II. Similarly, a significant reduction in HDL-cholesterol was observed in all age groups ($P<0.01$) and the maximum reduction was observed in age group IV (24.607±4.343 mg/dL) (Figure 7.1). In the case of triglycerides and VLDL cholesterol the significant increase was noted only in age groups III and IV (Table 7.1).

When anti-*H. pylori* IgG positive and negative sera analysed separately, the level of total cholesterol was decreased significantly ($P<0.01$) in anti- *H. pylori* IgG positive sera only (except in group II and group V) (Figures 7.2 and 7.3). Although there was a reduction in the total cholesterol in anti-*H. pylori* IgG negative sera, this reduction was not significant when compared to the control sera.

The HDL-cholesterol was significantly reduced ($P<0.01$) both in anti-*H. pylori* IgG positive and negative serum samples.

When the LDL-cholesterol was analysed in anti-*H. pylori* IgG positive and negative sera (Figure 7.2 and 7.3), it was observed that there was a significant reduction in LDL-cholesterol in anti-*H. pylori* IgG positive sera as compared to the control ($P<0.01$), except in age group V, and this significant reduction was not observed in anti-*H. pylori* IgG negative serum samples (Table 7.2 and 7.3).

Table 7.1 Lipid profile in gastric cancer patient sera

Age Group	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	Triglyceride (mg/dL)	VLDL Cholesterol (mg/dL)	LDL Cholesterol (mg/dL)
Group I (n=10)	149.137± 35.194	29.140± 5.006	163.793± 52.602	32.758± 10.520	87.238± 32.089
Group II (n=12)	177.695± 42.081	29.341± 4.957	154.019± 47.098	30.803± 9.419	117.550± 42.796
Group III (n=24)	158.148± 30.992	26.494± 6.089	180.578± 57.047	36.115± 11.409	95.456± 27.930
Group IV (n=23)	151.777± 30.253	24.607± 4.343	174.32± 65.581	34.864± 13.116	92.306± 27.389
Group V (n=10)	144.969± 45.293	25.251± 5.846	152.852± 36.447	30.570± 7.289	89.147± 36.102
Control (n=30)	178.120± 27.392	39.775± 7.177	132.339± 24.135	26.447± 4.843	117.685± 20.856

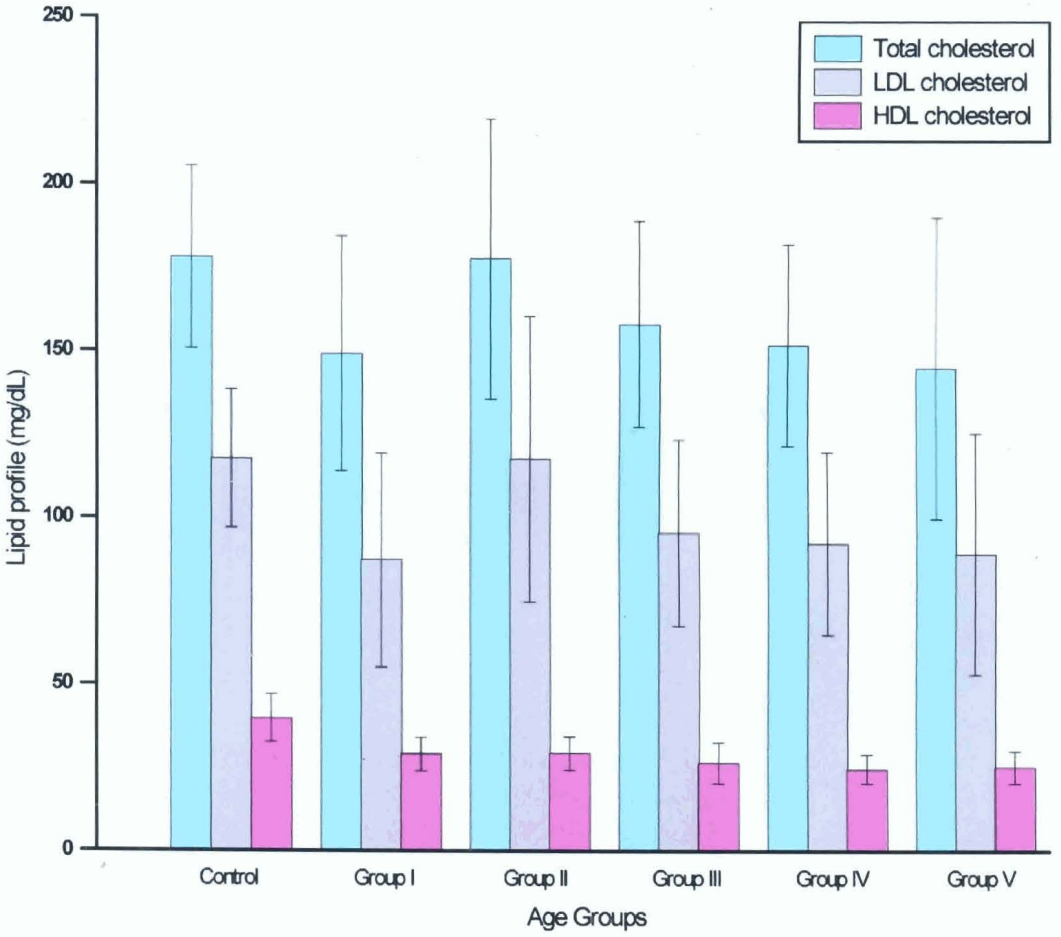

Figure 7.1 Levels of total cholesterol, LDL cholesterol and HDL cholesterol in gastric cancer patients

Table 7.2 Lipid profile in anti-*H. pylori* IgG positive gastric cancer patient sera

Age Group	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	Triglycerides (mg/dL)	VLDL Cholesterol (mg/dL)	LDL Cholesterol (mg/dL)
Group I (n=8)	143.200± 30.267	29.547± 5.583	144.623± 30.969	28.924± 6.193	84.727± 25.426
Group II (n=8)	176.603± 34.446	30.673± 4.469	135.023± 44.977	27.004± 8.995	118.925± 38.667
Group III (n=15)	155.592± 31.062	25.813± 5.029	190.975± 54.521	38.195± 10.904	91.585± 33.414
Group IV (n=16)	149.733± 29.157	24.745± 4.830	185.420± 73.528	37.084± 14.705	87.904± 25.437
Group V (n=7)	148.164± 51.528	24.220± 4.300	160.458± 41.385	32.091± 8.277	91.852± 41.997
Control (n=30)	178.12± 27.392	39.775± 7.177	132.339± 24.135	26.447± 4.843	117.685± 20.856

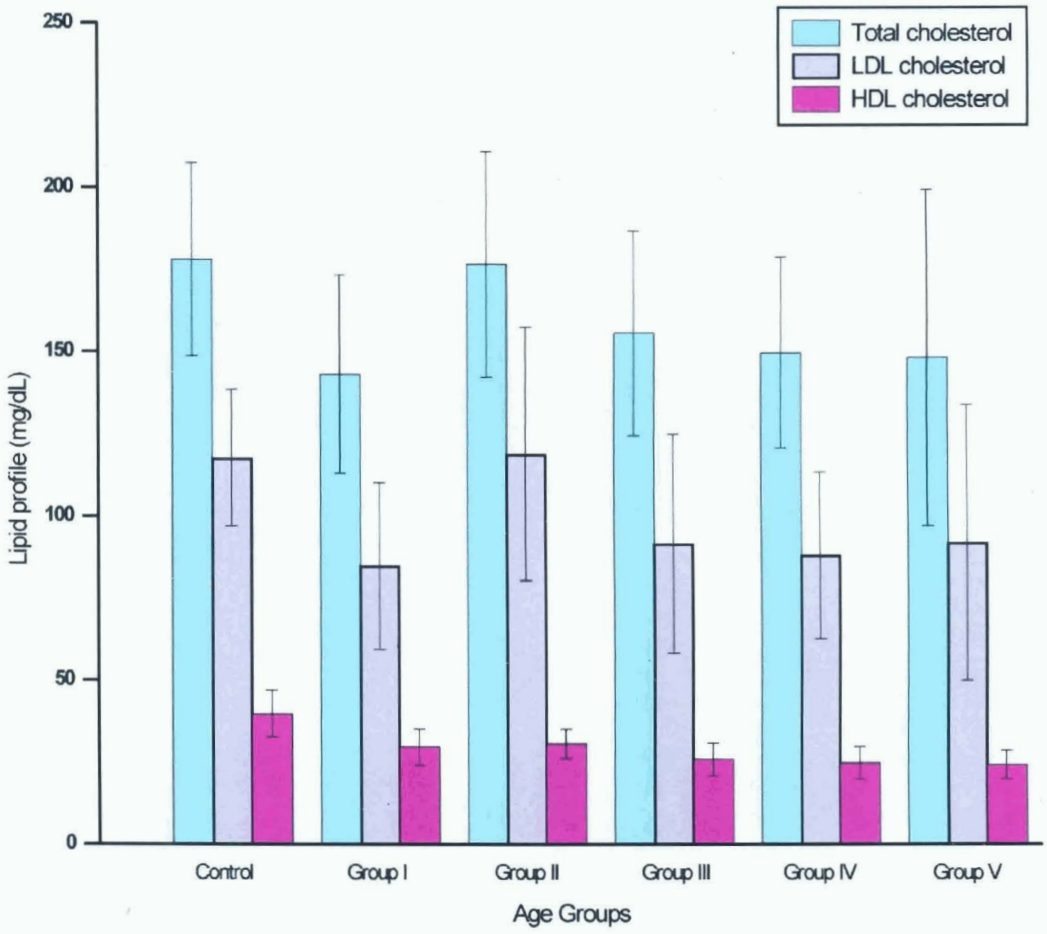

Figure 7.2 Levels of total cholesterol, LDL cholesterol and HDL cholesterol in anti-*H. pylori* IgG positive gastric cancer patients

Table 7.3 Lipid profile in anti-*H. pylori* IgG negative gastric cancer patient sera

Age Group	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	Triglycerides (mg/dL)	VLDL Cholesterol (mg/dL)	LDL Cholesterol (mg/dL)
Group I (n=2)	172.88± 57.66	27.51± 2.474	240.47± 59.08	48.094± 11.817	97.26± 66.97
Group II (n=4)	179.88± 60.946	26.677± 5.412	192.01± 22.95	38.402± 4.59	114.80± 56.57
Group III (n=9)	162.408± 32.254	27.628± 7.748	163.244± 60.112	32.648± 12.022	101.909± 34.293
Group IV (n=7)	156.451± 34.555	24.294± 3.266	148.947± 34.268	29.789± 6.853	102.367± 31.035
Group V (n=3)	137.513± 33.861	27.656± 9.271	135.103± 12.833	27.02± 2.566	82.83± 22.099
Control (n=30)	178.120± 27.392	39.775± 7.177	132.339± 24.135	26.447± 4.843	117.685± 20.856

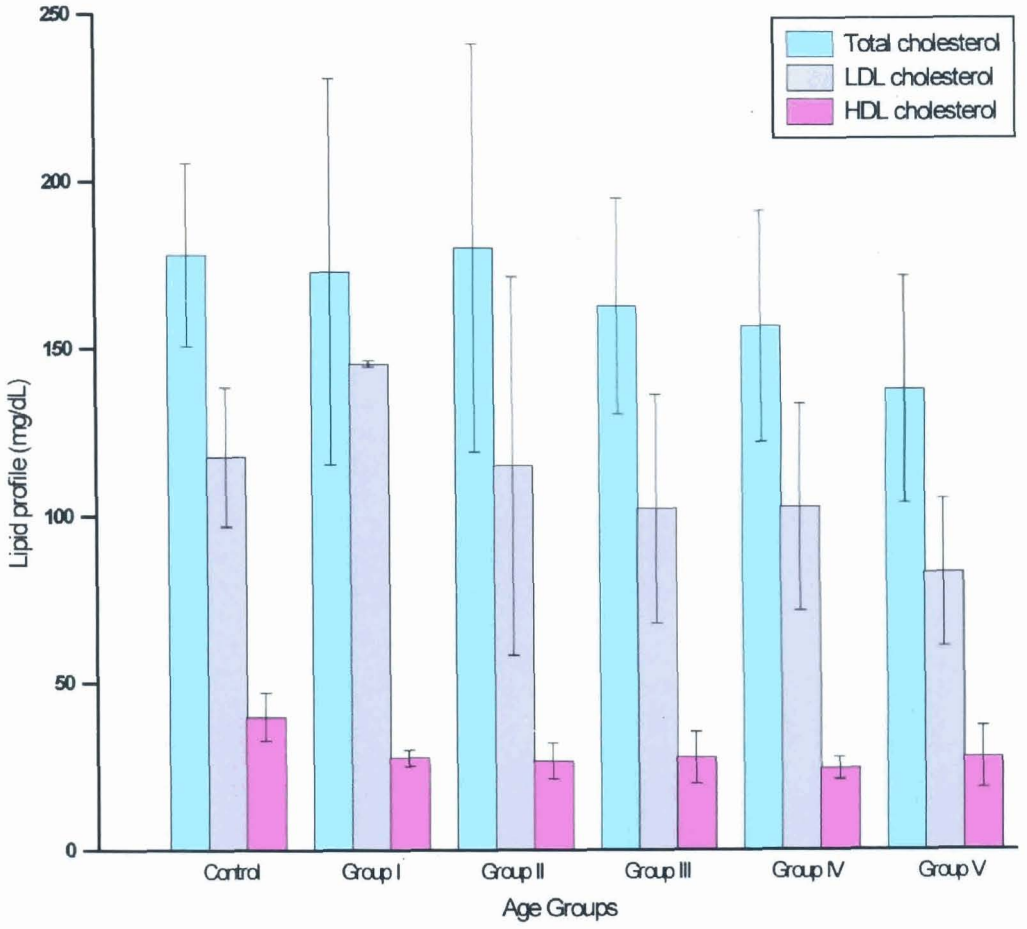

Figure 7.3 The levels of total cholesterol, LDL cholesterol and HDL cholesterol in anti-*H. pylori* IgG negative gastric cancer patients

DISCUSSION

The mean concentrations of total cholesterol, HDL-cholesterol and triglycerides in control samples were within the normal range.

It was observed 72.15% of gastric cancer patient sera were positive for anti-*H. pylori* IgG antibodies (Chapter V), and the lipid profile in anti-*H. pylori* IgG positive and negative sera were analysed and compared with the control.

There was a significant reduction ($P<0.01$) in total cholesterol, HDL-cholesterol and LDL-cholesterol in all age groups of anti-*H. pylori* IgG positive serum samples, except in age group II (Figure 7.2). There are several reports showing lower serum cholesterol levels in cancer patients (Beaglehole et al., 1980; Williams et al., 1981; Rose and Shipley, 1980). Similar observations were also made by Alexopoulose et al. (1987) in all types of cancers studied, except breast cancer.

Analysis of lipid profile in anti-*H. pylori* IgG negative sera showed that there was no significant reduction in the total cholesterol level when compared to the normal sera (Figure 7.3). A significant reduction in total cholesterol was noted in all age groups of anti-*H. pylori* IgG positive sera, except in age groups II and V, although a pattern of reduction was noted in these age groups. The level of LDL-cholesterol was significantly reduced ($P<0.01$) in anti-*H. pylori* IgG positive patients, while anti-*H. pylori* IgG negative patients did not showed significant reduction.

It was generally observed that *H. pylori* infection alter the lipid profile in non-cancerous patients. Chimienti et al. (2003) observed increased lipid profile in *H. pylori* infected subjects. Laurila et al. (1999) also noted higher levels of total cholesterol, HDL-cholesterol and triglycerides in *H. pylori* positive male patients when compared to the males with no signs of infection. However, no data is available in the literature regarding the lipid profile in *H. pylori* positive gastric cancer patients.

Cancer cells require more cholesterol than normal cells. This requirement may be fulfilled by higher hydroxymethyl glutaryl CoA reductase activity, the rate-limiting enzyme in the biosynthesis of cholesterol, or LDL-receptor expression or both. Caruso et al. (2002) observed significantly higher levels of hydroxymethyl glutaryl CoA reductase activity in neoplastic tissue than normal mucosa and LDL receptor expression dependent on the phenotype of the neoplastic tissue. They observed significantly higher expression of LDL receptors in intestinal type gastric cancer tissue than in diffuse type gastric cancer tissue. Vitols et al. (1985) showed that leukaemia cells from patients with acute myelogenous leukaemia have elevated levels of LDL-receptor activity. Later studies revealed the increased receptor mediated uptake of plasma LDL-cholesterol by human leukaemia cells (Vitols et al., 1990).

de Sanjose et al. (1996) observed reduced levels of serum cholesterol and triglycerides in stomach cancer patients before and after surgery. On the contrary, an Indian study revealed elevated levels of total cholesterol, free-cholesterol and HDL-cholesterol in plasma and erythrocyte membranes (Manoharan et al., 1997) in stomach cancer patients and it was speculated that this increase may be due to the life style of subjects such as alcohol consumption and cigarette smoking.

LDL (low density lipoprotein) carries about 70% of total plasma cholesterol to the cells and very little triglycerides. HDL (high density lipoprotein) contains about 20-30% of plasma cholesterol (Rifai et al., 1999). In the present study, decreased levels of LDL- and HDL-cholesterols and an increased triglyceride level was observed when compared to the normal control. Lowered levels of LDL- and HDL-cholesterols were reported in cancer patients previously (Alexopoulose et al., 1987). Many studies showed relatively unchanged levels of triglycerides in cancer patients (Nydegger and Butler, 1972; Alexopoulose et al., 1987). There was no significant variation in the levels of triglycerides in anti-*H. pylori* IgG positive and negative gastric cancer patient sera.

CONCLUSIONS

CONCLUSIONS

1. The present study revealed that about 54% of the gastro duodenal pathologies in patients were due to *H. pylori* infection, as assessed by rapid urease test. *H. pylori* were successfully isolated from human gastric biopsies of the patients. The isolation of *H. pylori* from the specimens has important clinical significance. Presently, gastric ulcer and associated conditions were proved to be infectious disease and the organism survives in the peculiar ecological niche in the stomach is *H. pylori*. Different mechanisms through which the organism develops gastric disorders including ulcer and cancer were elucidated. So treatment for gastric ulcers and associated conditions were switched to antimicrobial therapy, rather than therapy with agents for curing the symptoms. But rate of treatment failures were increased due to the increasing antimicrobial resistance developed by *H. pylori*.

2. The antimicrobial resistance in *H. pylori* may be either primary resistance or acquired resistance during the progression of infection. Both sensitive and resistant strains to antimicrobials have been isolated from the same patients (Loivukene et al., 2000). These points to the necessity of antimicrobial susceptibility testing of *H. pylori* strains isolated from each patient before commencing the treatment, thus the chance of treatment failures can be avoided. The method of isolation described in the present study is very simple and even clinical laboratories can isolate *H. pylori* from human biopsies provided they have facilities for microaerophilic condition and trained microbiologist for the purpose.

3. The protein profile analysis of the clinical isolates of *H. pylori* revealed strain variations. Depending on the production of VacA and CagA, *H. pylori* strains were grouped into type I strains and type II strains, of which the type I strains are the most virulent form of the organism. The clinical outcome of the infection depends on the type of strain causing the infection. One of the easy non-invasive methods for

the diagnosis of *H. pylori* infection is the serological detection of anti-*H. pylori* antibodies in patients. Since, IgA and IgM antibody detections have no clinical significance in *H. pylori* infection, anti-*H. pylori* IgG detection is mostly employed. The subclasses of human IgG and the preferential induction of these subclasses against different types of antigens were established. To find out the most useful antigen for the detection of anti-*H. pylori* IgG, *in vitro* proliferation and IgG subclass secretion by tonsillar mononuclear cells in response to the antigens prepared from isolate strains of *H. pylori* were performed.

4. In the present study, no significant *in vitro* cell proliferation using tonsillar mononuclear cells was observed when cells were stimulated with *H. pylori* antigens. Low biological and immunological activities of *H. pylori* lipopolysaccharides were reported. The proteins, such as VacA produced by *H. pylori* can inhibit mononuclear cell proliferation (Gebert et al., 2003; Knipp et al., 1996). Since the antigens used were not purified, it may contain components like VacA, which may inhibit cell proliferation.

5. All protein antigens used in the present study induced higher levels of IgG, except whole cell sonicate antigens of all strains and acid-glycine extract and n-octyl-β-D-glucopyranoside antigens of isolate strain 3. The predominant subclasses in these secretions were IgG_1 and IgG_3, even though there was a reduction in IgG_1 secretion when compared to the control. The inductions of IgG_1 and IgG_3 in response to protein antigens were well established. Thus, the results obtained with *H. pylori* antigens were consistent with the previous reports (Sundqvist et al., 1984). The decrease in IgG secretion in presence of the antigens of some isolates reflects the strain variation among the isolates.

6. The phenol-water extract antigens caused increased secretion of IgG by tonsillar mononuclear cells *in vitro*. The predominant subclass in these secretions was IgG_2

when compared to control. This finding is in agreement with the observations that carbohydrate antigens of bacterial pathogens preferentially induce IgG_2 secretion (Mayumi et al., 1983). Phenol-water extraction method is commonly used for the isolation of lipopolysaccharides from bacterial cell walls (Westphal and Jann, 1965).

7. The study on secretion of IgG subclasses by tonsillar mononuclear cells in response to *H. pylori* antigens has much clinical significance, because most of the serological methods for the diagnosis of *H. pylori* infection detect anti-*H. pylori* IgG. Many products available for this purpose show variability in sensitivity and specificity due to the type of antigens employed. The present study reveals that n-octyl-β-D-glucopyranoside extract antigens were most useful, since it induced maximum level of IgG *in vitro*.

8. The *H. pylori* acquisition occurs early in the childhood and the infection is chronic in nature. So early detection and eradication of the organism will be useful for preventing the development of gastro duodenal pathologies and gastric cancer. But invasive procedures like endoscopy will not be possible in children. So non-invasive methods like serology is useful for this purpose. There are reports showing the differences in the initial humoral response against *H. pylori* infection in children and adults. Mostly, initial antibody production in children was directed against low molecular weight antigens of *H. pylori*, while it was against high molecular weight antigens in adults (Mitchell et al., 1996). The present study showed that acid-glycine extract antigens mainly contain low molecular weight proteins and n-octyl-β-D-glucopyranoside preparations contain intense bands of high molecular weight proteins. So it was speculated that purified specific proteins of acid-glycine extract and n-octyl-β-D-glucopyranoside extract would be useful for detecting anti-*H. pylori* IgG in children and in adults with more sensitivity.

9. The analysis of IgG subclasses in gastric cancer patient sera has immense significance since the association of *H. pylori* infection in the development of gastric cancer was proved undoubtedly. 72% prevalence of *H. pylori* infection was detected in sera of gastric cancer patients by the analysis of anti-*H. pylori* IgG. The mean IgG_1 level was decreased in all age groups and the maximum decrease was observed in age group I. In contrast, the mean IgG_2 level was decreased in all age groups, except group V of the patients. Therefore, there was no significant variation in total IgG concentrations. The maximum increase was observed in age group IV. These findings were in agreement with the earlier observations of this laboratory (Anilkumar, 1993). When anti-*H. pylori* IgG positive and negative patients were analyzed separately, a reverse pattern in the levels of IgG_1 and IgG_2 observed. There was no significant variation in the levels of IgG_3 and IgG_4 in different age groups of the patients.

10. Increased IgG_2 production occurs in human body in response to carbohydrate antigens (Siber et al. 1980). *In vitro* studies with the antigen preparations from clinical isolate strains of *H. pylori* showed that phenol-water extract antigens caused increased production of IgG_2 by tonsillar mononuclear cells, when compared to other antigen preparations. So it was speculated that carbohydrate antigens, including lipopolysaccharide antigens of *H. pylori*, might induce the significant proportion of IgG_2 in gastric cancer patients. But the patterns of change in IgG subclasses were similar in anti-*H. pylori* IgG positive and negative sera. Therefore, there may be other factors that contribute to the secretion of elevated levels of IgG_2 subclass in gastric cancer patients. More over, the analysis of IgG subclasses was not specific to *H. pylori* antigens.

11. All *H. pylori* isolate strains induced increased murine macrophage activation, when the cells were stimulated with different antigen preparations. Increased levels of

nitric oxide (NO) generation and lysosomal enzyme activities were the markers used for macrophage activation. The data showed that many antigens caused more than 1.5-fold increase in the activation of macrophages. Phenol-water extracts showed the least induction of NO in the stimulated macrophages. The lower biological activities of *H. pylori* lipopolysaccharides were noticed (Perez-Perez et al., 1995). Mai et al. (1991) demonstrated the lipopolysaccharide independent mononuclear cell activation by surface proteins of *H. pylori*.

12. The present data showed increased levels of lysosomal enzymes in antigen-stimulated macrophages. The increase of more than 40 hydrolytic enzymes in activated macrophages was reported (Meltzer and Nacy, 1989). Thus the present study supports the enhanced activation of inflammatory cells in *H. pylori* infection and this could be one of the major pathogenic processes in the manifestation of clinical outcomes of *H. pylori* infection.

13. In the context of the reports showing increased levels of lipid profile in *H. pylori* infected non-cancerous patients (Chimienti et al., 2003) the analysis of lipid profile in gastric cancer patients with respect to *H. pylori* infection becomes significant. The gastric cancer patients showed a significant reduction in total cholesterol in all age groups, except group II ($P<0.05$). The reduction was maximum in age group IV. HDL- and LDL-cholesterol were also decreased in gastric cancer patients. But increased levels of triglycerides and VLDL-cholesterol were observed in the patients.

14. Anti-*H. pylori* IgG positive and negative serum sample analysis separately showed similar pattern in the lipid profile. But the reduction of total cholesterol and LDL-cholesterol were not significant in anti-*H. pylori* IgG negative serum samples ($P<0.01$). The reduction in HDL-cholesterol was independent of the presence or absence of anti-*H. pylori* IgG in the patients.

15. The increased uptake of cholesterol by transformed cells could explain the hypocholesterolemia observed in the tumour patients. This increased uptake may be via increased expression of LDL-receptors and hydroxymethyl glutaryl CoA reductase activity in tumour cells (Caruso et al., 2002; Vitols et al., 1990). Enhanced uptake of oxidized-LDL by macrophages was reported (Steinberg and Witztum, 1990) and many events leading to the oxidation of LDL were documented in *H. pylori* infections, such as increased levels of reactive oxygen species. Thus, it was speculated that the pronounced decrease in the total cholesterol and LDL cholesterol in anti-*H. pylori* IgG positive gastric cancer patients may be due to the increased inflammatory response in *H. pylori* infected persons. Further studies are required to find out the cause of pronounced reduction of total cholesterol and other lipids in the *H. pylori* positive gastric cancer patients.

REFERENCES

REFERENCES

Aalberse, R.C., R. van der Gaag and J. van Leeuwen. 1983. Serologic aspects of IgG_4 antibodies. *J. Immunol.* **130**: 722-726.

Abraham, P., N. Sindhu, and S.R. Naik. 1997. *In vitro* sensitivity of *Helicobacter pylori* in India. *Indian. J. Gastroenterol.* 16 (suppl. 1): S20-S21.

Adachi, M., T. Sekine, K. Imai, A. Yachi, and S. Sato. 1987. Serological and immunohistochemical detection of a gastrointestinal Cancer-associated asialoglycoprotein antigen of human with murine monoclonal antibody. *Jpn. J. Cancer Res.* **78**: 1370-1377.

Ahmed, A., D. Smoot, G. Littleton, R. Tackey, C.S. Walters, F. Kashanchi, C.R. Allen, and H. Ashktorab. 2000. *Helicobacter pylori* inhibits gastric cell cycle progression. *Microbes and Infection* **2**: 1159-1169.

Al-Assi, M.T., K. Miki, J.H. Walsh, D.P. Graham, M. Asaka, and D.Y. Graham. 1999. Non-invasive evaluation of *Helicobacter pylori* therapy: role of fasting on postprandial gastrin, pepsinogenI, pepsinogen II, or serum IgG antibodies. *Am. J. Gastroenterol.* **94**: 2367-2372.

Alexopoulose, C.G., B. Blatsios, and A. Avgerinos. 1987. Serum lipids and lipoprotein disorders in Cancer patients. *Cancer* **60**: 3065-3070.

Alkout, A.M., C.C. Blackwell, and D.M. Weir. 2000. Increased inflammatory responses of persons of blood group O to *Helicobacter pylori*. *J. Infect. Dis.* **181**: 1346-1349.

Allen, A., G. Flemstrom, A. Garner, and E. Kivilaakso. 1993. Gastroduodenal mucosal protection. *Physiol. Rev.* **73**: 823-857.

Alm, R.A., L.-S. Lee, D.T. Moir, B.L. King, E.D. Brown, P.C. Doig, D.R. Smith, B. Noonan, B.C. Guild, B.L. deJonge, G. Carmel, P.J. Tummino, A. Caruso, M. Urai-Nickelsen, D.M. Mills, C. Ives, R. Gibson, D. Merberg, S.D. Mills, Q. Jiang, D.E. Taylor, G.F. Vovis, and T.J. Trust. 1999. Genomic sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* **397**: 176-180.

Andersen, L.P., and T. Wadstrom. 2001. Basic bacteriology and culture. p. 27-38. In *Helicobacter pylori*: physiology and genetics eds. H.L.T. Mobley, G.L. Mendz, and S.L. Hazell. ASM Press, Washington DC.

Anderson, J.C., E. Cheng, M. Roeske, P. Marchildon, J. Peacock, and R.D. Shaw. 1997. Detection of serum antibodies to *Helicobacter pylori* by an immunochromatographic method. American J. *Gastroenterol.* **92**: 1135-1139.

Anilkumar, K.K. 1993. Some immunological and biochemical studies related to human gastric cancer. Ph. D. Thesis, Univestiy of Calict, Kerala, India

Anonymous. 1994. Live flukes and *Helicobacter pylori*. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Lyon, 7-14 June 1994. IARC Monogr. Eval. Carcinog. Risks. Hum. **61**: 1-241.

Anti, M., A. Armuzzi, E. Iscone, A. Valenti, M.E. Lippi, M. Covino, F.M. Vecchio, A. Buzzi, G. Pignataro, F. Bonvicini, and G. Gasbarrini. 1998. Epithelial cell apoptosis and proliferation in *Helicobacter pylori* related chronic gastritis. Itl. *J. Gastroenterol. Hepatol.* **30**: 153-159.

Appelmelk, B.J., B. Shiberu, C. Trinks, N. Tapsi, P.Y. Zheng, T. Verboom, J. Maaskant, C.H. Hokke, W.E.C.M. Schiphorst, D. Blanchard, I.M. Simoons-Smit, D.H. van den Eijnden, and C.M.J.E. Vanderbrook-Grauls. 1998. Phase variation in *Helicobacter pylori* lipopolysaccharide. *Infect. Immun.* **66**: 70-76.

Archimandritis, A. J. Bitsikas, M. Tjivras, E. Anastasakou, N. Tsavaris, D. Kalogeras, P. Davaris, and A. Fertakis. 1993. Non-cardia gastric adenocarcinoma and *Helicobacter pylori* infection. *Itl. J. Gastroenterol.* **25**: 368-371.

Arcila, P.L., D. Freeman, H. Kleanthous, A.M. O'Dowd, S. Lewis, A.K. Turner, E.L. Rees, T.J. Tibbitts, J. Greenwood, T.P. Monath, and M.J. Darsley. 2002. Attenuated *Salmonella enterica* serovar *Typhi* expressing urease effectively immunizes mice against *Helicobacter pylori* challenge as part of a heterologous mucosal priming–parenteral boosting vaccination regimen. *Infect. Immun.* **70**: 5096-5106.

Asaka, M., A.R. Sepulveda, T. Sugiyama, and D.Y. Graham. 2001. Gastric *Cancer*. p. 481-498. In *Helicobacter pylori*: physiology and genetics eds. H.L.T. Mobley, G.L. Mendz, and S.L. Hazell. ASM Press, Washington DC.

Asaka, M., M. Kato, M. Kudo, M. Katagiri, K. Nishikawa, J. Yoshida, H. Takeda, and K. Miki. 1995. Relationship between *Helicobacter pylori* infection, atrophic gastritis, and gastric carcinoma in a Japanese population. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1): S7-S10.

Asaka, M., T. Kimura, M. Kato, M. Kudo, K. Miki, K. Ogoshi, T. Kato, M. Tatsutta, and D.Y. Graham. 1994. Possible role of *Helicobacter pylori* infection in early gastric Cancer development. *Cancer* **73**: 2691-2694.

Asaka, M., T. Kimura, M. Kudo, H. Takeda, S. Mitani, T. Miyazaki, K. Miki, and D.Y. Graham. 1992. Relationship of *Helicobacter pylori* to serum pepsinogens in an asymptomatic Japanese population. *Gastroenterol.* **102**: 760-766.

Ashorn, P., P.L. Lahde, T. Ruuska, and A. Makipernaa. 1994. Gastric lymphoma in an 11 year-old boy: A case report. *Med. Pediatr. Oncol.* **22**: 66-67.

Atanassov, C., L. Pezennec, J. d'Alayer, G. Grollier, B. Picard, and J.L. Fauchere. 2002. Novel antigens of *Helicobacter pylori* correspond to ulcer related antibody pattern of sera from infected patients. *J. Clin. Microbiol.* **40**: 547-552.

Atherton, J.C. and R.C. Spiller. 1994. The urea breath test for *Helicobacter pylori*. *Gut* **35**: 723-725.

Atherton, J.C., P. Cao, R.M. Peek, Jr., M.K. Tummuru, M.J. Blaser, and T.L. Cover. 1995. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. *J. Biol. Chem.* **270**: 17771-17777.

Atherton, J.C., P.M. Sharp, T.L. Cover, G. Gonzalez-Valencia, R.M. Peek, Jr. S.A. Thompson, C.J. Hawkey, and M.J. Blaser. 1999. Vacuolating cytotoxin (vacA) alleles of *Helicobacter pylori* comprise two geographically wide spread types, m1 and m2, and have evolved through limited recombination. *Curr. Microbiol.* **39**: 211-218.

Awakawa, T., T. Sugiyama, K. Hisano, M. Karita, and A. Yachi. 1995. Detection and identification of cagA of *Helicobacter pylori* by polymerase chain reaction. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1): S75-S78.

Ballieux, R.E., G.M. Bernier, K.T. Tominaga, F.W. Putnam. 1964. Gammaglobulin antigenic types defined by heavy chain determinants. *Science* **145**: 168-170.

Bamford, K.B., J. Bickley, J.S.A. Collins, B.T. Johnston, S. Potts, V. Boston, R.J. Owen, and J.M. Sloan. 1993. *Helicobacter pylori*-comparison of DNA fingerprints provides evidence for intrafamilial infection. *Gut* **34**: 1348-1350.

Bamford, K.B., X.J. Fan, S.E. Crowe, J.F. Leary, W.K. Gourley, G.K. Luthra, E.G. Brooks, D.Y. Graham, V.E. Reyes, P.B. Ernst. 1998. Lymphocytes in the human gastric mucosa during *Helicobacter pylori* infection have a T helper cell 1 phenotype. *Gastroenterol.* **114**: 482.

Banatvala, N., K. Mayo, F. Megraud, R. Jennings, J.J. Deeks, and R.A. Feldman. 1993. The cohort effect and *Helicobacter pylori*. *J. Infect. Dis.* **168**: 219-221.

Beaglehole, R., M.A. Foulkes, I.A.M. Prior, and E.F. Eyles. 1980. Cholesterol and mortality in New Zealand Maoris. *Br. Med. J.* **1**: 285-287.

Bermejo, F., D. Boixeda, J.P. Gisbert, J.M. Sanz, R. Canton, V. Defarges, and C.M. de Argila. 2000. Concordance between noninvasive tests in detecting *Helicobacter pylori* and potential use of serology for monitoring eradication in gastric ulcer. *J. Clin. Gastroenterol.* **31**: 137-141.

Best, L.M., S.J.O.V. van Zanten, G.S. Bezanson, D.J.M. Haldane, and D.A. Malatjalian. 1992. Serological detection of *Helicobacter pylori* by a flow microsphere immunofluorescence assay. *J. Clin. Microbiol.* **30**: 2311-2317.

Bielory, L., D.M. Kemeny, D. Richards, and M.H. Lessof. 1990. IgG subclass antibody production in human serum sickness. *J. Allerg. Clin. Immunol.* **85**: 573-577.

Birkholz, S., U. Knipp, C. Nietzki, R.J. Adamek, and W. Opferkuch. 1993. Immunological activity of lipopolysaccharide of *Helicobacter pylori* on human peripheral mononuclear blood cells in comparison to lipopolysaccharides of other intestinal bacteria. *FEMS Immunol. Med. Microbiol.* **6**: 317-324.

Bjorkholm, B. and N.R. Salama. 2003. Genomics of *Helicobacter*. *Helicobacter* **8** (suppl. 1): 1-7.

Blanchard, T.G., J.G. Nedrud, and S.J. Czinn. 2000. Immunity against *Helicobacter pylori*. p. 623-629. In effects of microbes on the immune system. eds. M.W. Cunnigham. and R.S. Fujinami. Lippincott Williams and Wilkins. Philadelphia.

Blanchard, T.G., J.G. Nedrud, E.S. Reardon, and S.J. Czinn. 1999. Qualitative and quantitative analysis of the local and systemic antibody response in mice and humans with Helicobacter immunity and infection. *J. Infect. Dis.* **179**: 725-728.

Blaser, M.J. 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. *J. Infect. Dis.* **161**: 626-633.

Blaser, M.J. 1992. *Helicobacter pylori*: Its role in disease. *Clin. Infect. Dis.* **15**: 386-393.

Blaser, M.J. 1994. *Helicobacter pylori* phenotypes associated with peptic ulceration. *Scand. J. Gastroenterol.* **29** (suppl 205): 1-5.

Blasér, M.J. 1996. The bacteria behind ulcers. *Scientific American.* **274**: 104-107

Blaser, M.J. and J. Parsonnet. 1994. Parasitism by the "slow" bacterium *Helicobacter pylori* leads to altered gastric homeostasis and neoplasia. *J. Clin. Invest.* **94**: 4-8.

Blaser, M.J., P.H. Chyou, and A. Nomura. 1995. Age at establishment of *Helicobacter pylori* infection and gastric carcinoma, gastric ulcer, and duodenal ulcer risk. *Cancer Res.* **55**: 562-565.

Blecker, U., S. Lanciers, B. Hauser, D.I. Mehta, and Y. Vandenplas. 1995. Serology as a valid screening test for *Helicobacter pylori* infection in asymptomatic subjects. *Arch. Pathol. Lab. Med.* **119**: 30-32.

Bode, G., F. Mauch, H. Ditschuneit, and P. Malfertheiner. 1993. The coccoid forms of *Helicobacter pylori*- criteria for their viability. *Epidemiol. Infect.* **111**: 483-490.

Bodger, K. and J.E. Crabtree. 1998. *Helicobacter pylori* and gastric inflammation. *Br. Med. Bull.* **54**: 139-150.

Bolin, I., H. Lanroth, and A.M. Svennerholm. 1995. Identification of *Helicobacter pylori* by immunological dot blot method based on reaction of a species specific monoclonal antibody with a surface-exposed protein. *J. Clin. Microbiol.* **33**: 381-384.

Boneristiano, M., S.R. Paccani, S. Barone, C. Ulivieri, L. Patrussi, D. Ilver, A. Amedei, M.M. D'Elios, J.L Telford, and C.T. Baldari. 2003. The *Helicobacter pylori* vacuolating toxin inhibits T-cell activation by two independent mechanisms. *J. Exp. Med.* **198**: 1887-1897.

Bortran, R.F., and V. Vetvicka. 1995. Methods in cellular and molecular immunology. CRC press Inc. p 6-7.

Boyanova, L., I. Stancheva, Z. Spassova, N. Katzarov, I. Mitov, and R. Koumanova. 2000. Primary and combined resistance to four antimicrobial agents in *Helicobacter pylori* in Sofia, Bulgaria. *J. Med. Microbiol.* **49**: 415-418.

Breiter, J.R., D. Riff, T.J. Humphries for the rabeprazole study group. 2000. Rabeprazole is superior to ranitidine in the management of active duodenal ulcer disease: Results of a double blind, randomized North American study. *Am. J. Gastroenterol.* **95**: 936-942.

Brenner, H., G. Bode, and H. Boeing. 2000. *Helicobacter pylori* infection among offsprings of patients with stomach *Cancer. Gastroenterol.* **118**: 31-35.

Buck, G.E., W.K. Gourley, W.K. Lee, K. Subramanyam, J.M. Latimer, and A.R. DiNuzzo. 1986. Relation of *Campylobacter pyloridis* to gastritis and peptic ulcer. *J. Infect. Dis.* **153**: 664-669.

Burch, H.B. 1969. Acid phosphatase. p. 947-948. In Methods in Enzymology Vol. III.eds. P. Colowick and N.O. Kaplan. Academic Press. NewYork.

Buria, M.K., O. Parkash, B. Joshi, K.K. Mohanty, K. Kaloch, and U. Sengupta. 1998. Levels of IgG subclasses in active and inactive cases in the disease spectrum of leprosy. *Int. Arch. Allergy Immunol.* **115**: 61-66.

Burne, R. and Y.Y.M. Chen. 2000. Bacterial ureases in infectious diseases. *Microb. Infect.* **2**: 533-542.

Butch, A.W., K.A. Macke, M.G. Scott, M. Inkster, and M.H. Nahm. 1989. Mitogen-induced human IgG subclass expression. II. IgG_1 and IgG_3 subclasses are preferentially stimulated by a combination of *Staphylococcus aureus* Cowan I and pokeweed mitogen. *Human Immunol.* **24**: 207-218.

Byrd, J.C., C.K. Yanker, Q.S. Xu, L.R. Sternberg, and R.S. Bresalier. 2000. Inhibition of gastric mucin synthesis by *Helicobacter pylori*. *Gastroenterol.* **118**: 1072-1079.

Byrne, M.F., J.F. Murphy, P.A. Concoran, J.C. Atherton, K.M. Sheehan, D. Cox, F.E. Murray, and D.J. Fitzgerald. 2003. Helicobacter pylori induces cyclooxygenase-1 and cyclooxygenase-2 expression in vascular endothelial cells. *Scand. J. Gastroenterol.* **38**: 1023-1030.

Calvert, R., J. Randerson, P. Evans, L. Cawkwell, F. Lewis, M.F. Dixon, A. Jack, R. Owen, C. Shiach, and G.J. Morgan. 1995. Genetic abnormalities during transition from *Helicobacter pylori* associated gastritis to low-grade MALToma. *Lancet* **345**: 26-27.

Cantorna, M.T., and E. Balish. 1990. Inability of human clinical strains of *Helicobacter pylori* to colonize the alimentary tract of rodents. *Can. J. Microbiol.* **36**: 237-241.

Caruso, M.G., M. Notarnicola, A. Cavallini, and A. DiLeo. 2002. 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and low-density lipoprotein receptor expression in diffuse type and intestinal type human gastric Cancer. *J. Gastroenterol.* **37**: 504-508.

Cassaro, M., M. Rugge, O. Gutierrez, G. Leandro, D.Y. Graham, R.M. Genta. 2000. Topographic patterns of intestinal metaplasia and gastric Cancer. *Am. J. Gastroenterol.* **95**: 1431-1438.

Casswal, T.H., S.A. Sarker, M.J. Albert, G.J. Fuchs, M. Bergstrom, L. Bjorck, and L. Hammarstrom. 1998. Treatment of *Helicobacter pylori* infection in infants in rural Bangladesh with oral immunoglobulins from hyperimmune bovine colostrums. *Aliment. Pharmacol. Ther.* **12**: 563-568.

Caudron, P.E., and D.F. Kirby. 1989. Comparison of rapid urease tests, staining techniques, and growth on different solid media for detection of *Campylobacter pylori*. *J. Clin. Microbiol.* **27**: 1527-1530.

Cellini, L., N. Allocati, D. Angelucci, T. Iezzi, E. D. Campli, L. Marzio, and B. Dainelli. 1994. Coccoid *Helicobacter pylori* not culturable *in vitro* reverts in mice. *Microbiol. Immunol.* **38**: 843-850.

Ceponis, P.J., D.M. McCay, R.J. Manaker, E.G. Mata, and N.L. Jones. 2003. *Helicobacter pylori* infection interferes with epithelial stat6-mediated interleukin-4 signal transduction independent of cagA, cage, and vacA. *J. Immunol.* **171**: 2035-2041.

Chan, F.K.L., J.J.Y. Sung, Y.T. Lee, W.K. Leung, L.Y. Chan, M.Y. Yung, S.C.S. Chung. 1997. Does smoking predisposes to peptic ulcer relapse after eradication of *Helicobacter pylori*? *Am. J. Gastroenterol.* **92**: 442-444.

Chan, W.Y., P.K. Hui, K.M. Leung, J. Chow, F. Kwok, and C.S. Ng. 1994. Coccoid forms of *Helicobacter pylori* in the human stomach. *Am. J. Clin. Pathol.* **102**: 503-507.

Chi, D.S., D. Shen, C. Li, T. Ha, D. Ferguson, and E. Thomas. 1994. TNF-α and nitric oxide (NO) synthase gene expressions in cultured macrophages enhanced by soluble proteins from *Helicobacter pylori*. *FASEB J.* **8**: A253-A255.

Chimienti, G., F. Russo, B.L. Lamanuzzi, M. Nardulli, C. Messa, A. DiLeo, M. Correale, V. Gianuzzi, and G. Pepe. 2003. *Helicobacter pylori* is associated with modified lipid profile: impact on Lipoprotein (a). *Clin. Biochem.* **36**: 359-365.

Ching, C.K. and S.K. Lam. 1994. *Helicobacter pylori* in relation to peptic ulcer and gastric *Cancer* in South and North China. *J. Gastroenterol. Hepatol.* **9**: S4-S7.

Christensen, W.B. 1946. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from Salmonella and Shigella types. *J. Bacteriol.* **52**: 461-466.

Claeys, D., G. Faller, B.J. Appelmelk, R. Nigrini, and T. Kirchner. 1998. The gastric H^+, K^+-ATPase is the major autoantigen in chronic *Helicobacter pylori* gastritis with body mucosa atrophy. *Gastroenterol.* **115**: 340-347.

Coligan, E., A.M. Kruisheck, D.H. Margulier, E.M. Shevach and W. Strober. 1996. Current protocols in immunology. Vol. I, II, and III. Unit 7 and 14. John Wiley and Sons, Inc. U.S.

Collins, J.S.A. 1992. Role of *Helicobacter pylori* in gastritis and duodenitis in man. Agents Actions, special conference issue C47.

Correa, P. 2000. A new paradigm for human *Carcinogenesis*. *J. Clin. Gastroenterol.* **30**: 341-342.

Coutelier, J.-P., J.T.M. van der Logt, F.W.A. Heessen, A. Vink, and J. van Snick. 1988. Virally induced modulation of murine IgG antibody subclasses. *J. Exp. Med.* **168**: 2373-2378.

Cover, T.L. and M.J. Blaser. 1992. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J. Biol. Chem.* **267**: 10570-10575.

Cover, T.L., W. Puryear, G.I. Perez-Perez, and M.J. Blaser. 1991. Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. *Infect. Immun.* **59**: 1264-1270.

Cover, T.L., Y. Glupczynski, A.P. Lage, A. Burette, M.K.R. Tummuru, G.I. Perez-Perez, and M.J. Blaser. 1995. Serologic detection of infection with $cagA^+$ *Helicobacter pylori* strain. *J. Clin. Microbiol.* **33**: 1496-1500.

Crabtree, J. 1994. Immunopathological aspects of *Helicobacter pylori* associated injury of the gastric mucosa. *Mol. Med.* **31**: 1340-1348.

Crabtree, J.E., J.I. Wyatt, G.M. Sobala, G. Miller, D.S. Tompkins, J.N. Primrose, and A.G. Morgan. 1993. Systemic and mucosal humoral responses to *Helicobacter pylori* in gastric *Cancer*. *Gut* **34**: 1339-1343.

Crabtree, J.E., N. Figura, J.D. Taylor, M. Bignoli, D. Armellini, and D.S. Tompkins. 1992. Expression of 120 kilodalton protein and cytotoxicity in *Helicobacter pylori*. *J. Clin. Pathol.* **45**: 733-734.

Crabtree, J.E., M.J. Mahony, J.D. Taylor, R.V. Heatley, J.M. Littlewood, and D.S. Tompkins. 1991. Immune responses to *Helicobacter pylori* in children with recurrent abdominal pain. *J. Clin. Pathol.* **44**: 768-771.

Crowe, S.E., L. Alwarez, M. Dytoc, R.H. Hunt, M. Muller, P. Sherman, J. Patel, Y. Jim, and P.B. Ernst. 1995. Expression of interleukin-8 and CD54 by human gastric epithelium after *Helicobacter pylori* infection *in vitro*. *Gastroenterol.* **108**: 65-74.

Cussac, V., R.L. Ferrero, and A. Labigne. 1992. Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen limiting conditions. *J. Bacteriol.* **174**: 2466-2473.

Dandekar, S., E. Reay, J.M. Taylor, and J.V. Solnik. 2003. Apoptosis of gastric lymphocytes in *Helicobacter pylori* infected rhesus macaques. *Dig. Dis. Sci.* **48**: 1073-1080.

Davenport, H.W. 1964. Gastric mucosal injury by fatty and acetylsialicylic acids. *Gastroenterol.* **46**: 245-252.

Davenport, H.W. 1970. Back-diffusion of acid through the gastric mucosa and its physiological consequences. *Prog. Gastroenterol.* **2**: 42-56.

Davies, G.R., C.E. Collins, N. Banatwala, M.T. Sheaff, Y. Abdi, L. Klements, and D.S. Rampton. 1993. A direct relationship between infective load of *Helicobacter pylori* and oxygen free radical production in antral mucosal biopsies. *Gut* **34** (suppl. 1): 73.

Day, A.S., N.L. Jones, J.T. Lynett, H.A. Jennings, C.A. Fallone, R. Beech, and P.M. Sherman. 2000. cagE is a virulence factor associated with *Helicobacter pylori*-induced duodenal ulceration in Children. *J. Infect. Dis.* **181**: 1370-1375.

de Bernard, M., D. Burroni, E. Papini, R. Rappuoli, J. Telford, and C. Montecucco. 1998. Identification of the *Helicobacter pylori* VacA toxin domain active in the cell cytosol. *Infect. Immun.* **66**: 6014-6016.

Debets-Ossenkopp, Y.J., M. Sparrius, J.G. Kusters, J.J. Kolkman, and C.M.J.E. Vanderbrook-Grauls. 1996. Mechanism of clarithromycin resistance in clinical isolates of *Helicobacter pylori*. *FEMS Microbiol. Lett.* **142**: 37-42.

DeBoer, W.A., R.J.X.M. van Etten, P.M. Schneeberger, and G.N.J. Tytgat. 2000. A single drug for *Helicobacter pylori* infection: First results with a new bismuth triple monocapsule. *Am. J. Gastroenterol.* **95**: 641-645.

DeRobertis, E.D.P. and E.M.F. DeRobertis, Jr. 1995. Cellular and molecular biology. 8^{rr}eds. p. 268. B.I. Waverly Edn. New Delhi.

de Sanjose, S., N. Munoz, G. Sobala, J. Vivas, S. Peraza, E. Cano, D. Castro, V. Sanchez, O. Adrade, D. Tompkins, C.J. Schorah, A.T. Axon, M. Benz, and W. Oliver. 1996. Antioxidants, *Helicobacter pylori*, and stomach *Cancer* in Venizuela. *Eur. J. Cancer Prev.* **5**: 57-62.

Dick, J.D. 1990. *Helicobacter* (*Campylobacter*) *pylori*: A new twist to an old disease. *Annu. Rev. Microbiol.* **44**: 249-269.

Dixon, M.F. 2001. Pathology of gastritis and peptic ulceration. p. 459-469. In *Helicobacter pylori*: physiology and geneticseds.H.L.T. Mobley, G.L. Mendz, and S.L. Hazell. ASM Press. Washington DC.

Doig, P. J.W. Austin, M. Kostrzynska, and T.J. Trust. 1992. Production of a conserved adhesin by the human gastroduodenal pathogen *Helicobacter pylori*. *J. Bacteriol.* **174**: 2539-2547.

Dore. M.P., H.M. Malaty, D.Y. Graham, G. Fanciulli, G. Delitala, and G. Realdi. 2002. Risk factors associated with *Helicobacter pylori* infection among children in a defined geographic area. *Clin. Infect. Dis.* **35**: 240-245.

Drake, I.M., M.J. Davies, N.P. Mapstone, M.F. Dixon, C.J. Schorah, K.L.M. White. D.M. Chalmers and A.T.R. Axon. 1996. Ascorbic acid may protect against human gastric *Cancer* by scavenging mucosal oxygen radicals. *Carcinogenesis* **17**: 559-562.

Drazek, E.S., A. Dubois, and R.K. Holmes. 1994. Characterization and presumptive identification of *Helicobacter pylori* isolates from rhesus monkeys. *J. Clin. Microbiol.* **32**: 1799-1804.

Dunn, B.E., G.P. Campbell, G.I. Perez-Perez, and M.J. Blaser. 1990. Purification and characterization of urease from *Helicobacter pylori*. *J. Biol. Chem.* **265**: 9464-9469.

Dunn, B.E., M. Altmann, and G.P. Campbell. 1991. Adherence of *Helicobacter pylori* to gastric carcinoma cells: Analysis by flow cytometry, *Reviews of Infectious Diseases* **13** (Suppl 8): S657-S664.

Dunn, B.E., R.M. Roop, C.C. Sung, S.A. Sharma, G.I. Perez-Perez, and M.J. Blaser. 1992. Identification and purification of a cpn60 heat shock protein homologs from *Helicobacter pylori*. *Infect. Immun.* **60**: 1946-1951.

Eaton, K.A., C.E. Cetrenich, K.M. Makin, and S. Krakowka. 1995. Virulence of coccoid and bacillary forms of *Helicobacter pylori* in gnotobiotic piglets. *J. Infect. Dis.* **171**: 459-462.

Eaton, K.A., D.R. Morgan and S. Krakowka. 1992. Motility as a factor in the colonization of gnotobiotic piglets by *Helicobacter pylori*. *J. Med. Microbiol.* **37**: 123-127.

Eaton, K.A., D.R. Morgan, C.L. Brooks, and S. Krakowka. 1991. Essential role of urease in the pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect. Immun.* **59**: 2470-2475.

Eaton, K.A., M. Mefford, and T. Thevenot. 2001. The role of T cell subsets and cytokines in the pathogenesis of *Helicobacter pylori* gastritis in Mice. *J. Immunol.* **166**: 7456-7461.

Eaton, K.A., T.L. Cover, M.K. Tummuru, M.J. Blaser and S. Krakowka. 1997. Role of vacuolating cytotoxin in gastritis due to *Helicobacter pylori* in gnotobiotic piglets. *Infect. Immun.* **65**: 3462-3464.

Eidt, S., M. Stolte, and R. Fischer. 1994. *Helicobacter pylori* gastritis and primary gastric non-Hodgkin's lymphomas. *J. Clin. Pathol.* **47**: 436-439.

Elitsur, Y., A. Reghuverra, T. Sadat, and P. Vaid. 2000. Is gastric nodularity a sign for gastric inflammation associated with *Helicobacter pylori* infection in children? *J. Clin. Gastroenterol.* **30**: 286-288.

El-Omar, E.M., K. Oien, L.S. Murray, A. El-Nujumi, A. Wirz, D. Gillen, C. Williams, G. Fullarton, and K.E.L. McColl. 2000. Increased prevalence of pre*Cancer*ous changes in relatives of gastric *Cancer* patients: Critical role of *H. pylori*. *Gastroenterol.* **118**: 22-30.

El-Zimaity, H.M., H. Ota, M.T. Al-Assi, R.M. Genta, and D.Y. Graham. 1995. Confirmation of successful therapy of *Helicobacter pylori* infection: number and site of biopsies or a rapid urease test. *Am. J. Gastroenterol.* **90**: 1962-1964.

El-Zimaity, H.M.T., D.Y. Graham, R.M. Genta, and J. Lechago. 2000. Sustained increase in gastric antral epithelial cell proliferation despite cure of *Helicobacter pylori* infection. *Am. J. Gastroenterol.* **95**: 930-935.

Estevans, J., P. Fidalgo, T. Tendeiro, C. Chagas, A. Ferra, C.N. Leitao, and F.C. Mira. 1993. Anti-*Helicobacter pylori* antibodies prevalence and gastric adenocarcinoma in Portugal: report of a case-control study. *Eur. J. Cancer Prevent.* **2**: 377-380.

Esumi, H., and S.R. Tannenbaum. 1994. US-Japan cooperative *Cancer* research programme: seminar on nitric oxide synthase and *Carcinogenesis*. *Cancer Res.* **54**: 297-301.

Eurogast study group. 1993. An international association between *Helicobacter pylori* infection and gastric *Cancer*. *Lancet* **341**: 1359-1362.

Evans, D.J. Jr., D.G. Evans, D.Y. Graham, and P.D. Klein. 1989. A sensitive and specific serologic test for detection of *Campylobacter pylori* infection. *Gastroenterology* **96**: 1004-1008.

Evans, D.J.Jr., D.G. Evans, T. Takemura, H. Nakano, H.C. Lampert, D.Y. Graham, D.N. Granger and P.R. Kvietys. 1995. Characterization of a *Helicobacter pylori* neutrophil-activating protein. *Infect. Immun.* **63**: 2213-2220.

Evans, Jr. D.J., D.G. Evans, L. Engstrand, and D.Y. Graham. 1992. Urease associated heat shock proteins of *Helicobacter pylori*. *Infect. Immun.* **60**: 2125-2127.

Everhart, J.E., D.K. Moran, G.I. Perez-Perez, T.S. Tralka, and G. McQuillan. 2000. Seroprevalence and ethnic differences in *Helicobacter pylori* infection among adults in the United States. *J. Infect. Dis.* **181**: 1359-1363.

Fagioli, F., G.M. Rigolin, A. Cuneo, G. Scalpoli, R. Spemadda, P. Cavazzini, and G. Castold. 1994. Primary gastric lymphoma: distribution and clinical relevance of different epidemiological factors. *Haematologica* **79**: 213-217.

Faller, G., H. Steininger, J. Kranzlein, H. Maul, T. Kerkau, J. Hensen, E.G. Hahn, and T. Kirchner. 1997. Antigastric autoantibodies in *Helicobacter pylori* infection: Implications of histological and clinical parameters of gastritis. *Gut* **41**: 619-623.

Fallone, C.A., A.N. Barkun, G. Friedman, S. Mayrand, V. Loo, R. Beech, L. Best, and L. Joseph. 2000. Is *Helicobacter pylori* eradication associated with gastroesophageal reflux disease? *Am. J. Gastroenterol.* **95**: 914-920.

Fallone, C.A., A.N. Burkun, M.U. Gottke, L.M. Best, V.G. Loo, S.V. van Zanten, T. Nguyen, A. Lowe, T. Fainsilber, K. Kouri, and R. Beech. 2000. Association of *Helicobacter pylori* genotype with gastroesophageal reflux disease and other upper gastrointestinal diseases. *Am. J. Gastroenterol.* **95**: 659-669.

Fangrat, D.K., M. Raeiszadeh, D. Dzierzanowska, M. G. Dura, C.D. Cedro, and J.E. Crabtree. 2003. IgG subclass response to *Helicobacter pylori* and CagA antigens in children. *Clin. Exp. Immunol.* **134**: 442-446.

Ferguson, D.A., L. Chuanfu, N.R. Patel, W.R. Mayberry, D.S. Chi, and E. Thomas. 1993. Isolation of *Helicobacter pylori* from saliva. *J. Clin. Microbiol.* **31**: 2802-2804.

Fernando, N., J. Holton, I. Zulu, D. Vaira, P. Mwaba, and P. Kelly. 2001. *Helicobacter pylori* infection in an urban African population. *J. Clin. Microbiol.* **39**: 1323-1327.

Ferrante, A., B. Rowan-Kelly, L.J. Beard, and G.M Maxwell. 1986. An enzyme linked immunosorbent assay for the quantitation of human IgG subclasses using monoclonal antibodies. *J. Immunol. Methods* **93**: 207-212.

Ferrero, R.L., S.L. Hazell, and A. Lee. 1988. The urease enzymes of *Campylobacter pylori* and a related bacterium. *J. Med. Microbiol.* **27**: 33-40.

Figueroa, G., M. Troncoso, M.S. Toledo, G. Faundez and R. Acuna. 2002. Prevalence of serum antibodies to *Helicobacter pylori* VacA and CagA and gastric disease in Chile. *J. Med. Microbiol.* **51**: 300-304.

Fiocca, R., O. Luinetti, L. Villani, A.M. Chiaravalli, C. Capella, and E. Solcia. 1994. Epithelial cytotoxicity, immune response and inflammatory components of *Helicobacter pylori* gastritis. *Scand. J. Gastroenterol.* **205** (suppl.):11-21.

Fishman, W.H., and P. Berbfield. 1969. β-glucouronidase. p. 262-264. In Methods in Enzymology Vol. I.eds.P. Colowick and N.O. Kaplan. Academic Press. NewYork.

Forman, D., D.G. Newell, F. Fullerton, J.W. Yarnell, A.R. Stacy, N. Wald, and F. Sitas. 1991. Association between infection with *Helicobacter pylori* and risk of gastric *Cancer*: evidence from a prospective investigation. *Br. Med. J.* **302**: 1302-1305.

Forne, M., J. Dominguez, F.F. Benares, J. Lite, M. Esteve, N. Gali, J.C. Epsinos, S. Quintana and J.M. Viver. 2000. Accuracy of an enzyme immunoassay for the detection of *Helicobacter pylori* in stool specimens in the diagnosis of infection and post-treatment check-up. *Am. J. Gastroenterol.* **95**: 2200-2205.

Foynes, S., N. Dorrell, S.J. Ward, R.A. Stabler, A.A. McColm, A.N. Rycroft and B.W. Wren. 2000. *Helicobacter pylori* possesses two CheY response regulators and a histidine kinase sensor, CheA, which are essential for chemotaxis and colonization of the gastric mucosa. *Infect. Immun.* **68**: 2016-2023.

French, M. 1986. Serum IgG subclasses in normal adults. *Monogr. Allergy* 19: 100-107.

Friedewald, W.T., R.I. Levy, and D.S. Fredrickson. 1972. Estimation of the concentration of low-density lipoprotein concentration in plasma, without use of the preparative ultra centrifuge. *Clin. Chem.* **18**: 499-502.

Fu, S., K.S. Ramanujam, A. Wong, G.T. Fantry, C.B. Drachenberg, S.P. James, S.J. Meltzer, and K.T. Wilson. 1999. Increased cellular expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase2 in *Helicobacter pylori* gastritis. *Gastroenterol.* **116**: 1319-1329.

Fujioka, T., R.U. Uribe, T. Kubota, K. Murakami, H. Kawasaki and M. Nasu. 1995. Peptic ulcer recurrence after *Helicobacter pylori* eradication: a 5-year follow-up study. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1): S35-S38.

Fujioka, T., S. Honda and M. Tokieda. 2000. *Helicobacter pylori* infection and gastric carcinoma in animal models. *J. Gastroenterol. Hepatol.* **15** (suppl.): D55-D59.

Gebert, B., W. Fischer, E. Weiss, R. Hoffman, and R. Haas. 2003. *Helicobacter pylori* vacuolating cytotoxin inhibits T-lymphocyte activation. *Science* **301**: 1099-1102.

Geis, G., S. Suerbaum, B. Forsthoff, H. Leying, and W. Opferkuch. 1993. Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*. *J. Med. Microbiol.* **38**: 371-377.

Genta, R.M. 1995. *Helicobacter pylori* as a promoter of intestinal metaplasia and gastric *Cancer*: an alluring hypothesis in search of evidence. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1): S25-S30.

Genta, R.M., G.M. Lew, and D.Y. Graham. 1993. Changes in the gastric mucosa following eradication of *Helicobacter pylori* infection. *Mod. Pathol.* **6**: 281-289.

Ghiara, P., M. Marchetti, M.J. Blaser, M.K. Tummuru, T.L. Cover, E.D. Segal, L.S. Tompkins, and R. Rappuoli. 1995. Role of Helicobacter virulence factors vacuolating cytotoxin, CagA, and urease in the mouse model of disease. *Infect. Immun.* **63**: 4154-4160.

Gill, H.H., P. Majumdar, K. Sankaran, and H.G. Desai. 1994. Age-related prevalence of *Helicobacter pylori* antibodies in Indian subjects. *Indian J. Gastroenterol.* **13**: 92-94.

Glupczynski, Y., and the European multicentre study group on antibiotic susceptibility to *Helicobacter pylori*. 1992. Results of a multicentre European survey in 1991 of metronidazole resistance in *Helicobacter pylori*. *Eur. J. Clin. Microbiol. Infect. Dis.* **11**: 777-781.

Go, M.F. and D.Y. Graham. 1994. How does *Helicobacter pylori* cause duodenal ulcer disease: The bug, the host, or both? *J. Gastroenterol. Hepatol.* **9**: S8-S12.

Goddard, A and R. Logan. 1995. One-week low-dose triple therapy: new standards for *Helicobacter pylori* treatment. *Eur. J. Gastroenterol. Hepatol.* **7**: 1-3.

Gokeel, A., Y. Gumurdulu, F. Kayaselcuk, E. Serin, B. Ozer, A.K. Ozsahin, and N. Guvener. 2003. *Helicobacter pylori* has no effect on plasma ghrelin levels. *Eur. J. Endocrinol.* **148**: 423-426.

Goldstein, J.L., Y.K. Ho, S.K. Basu, and M.S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA* **76**: 333-337.

Goldstone, A.R., P. Quirke, and M.F. Dixon. 1996. *Helicobacter pylori* infection and gastric Cancer. *J. Pathol.* **179**: 129-137.

Goodman, J.W. 1987. Immunoglbulins I: Structure and function. p. 27-36. In D.P.Stites. J.D. Stobo, J.V. Wells, eds. Basic and Clinical immunology. 6theds.Los Altos, CA: Appelton and Lange publications.

Goodwin, C.S. 1993. Gastric *Cancer* and *Helicobacter pylori*: the whispering killer. *Lancet* **342**: 507-508.

Goodwin, C.S. 1994. How *Helicobacter pylori* acquired its name, and how it overcomes gastric defense mechanisms. *J. Gastroenterol. Hepatol.* **9**: S1-S3.

Goodwin, C.S. and J.A. Armstrong. 1990. Microbiological aspects of *Helicobacter pylori* (*Campylobacter pylori*). *Eur. J. Clin. Microbiol. Infect. Dis.* **9**: 1-13.

Goodwin, C.S., J.A. Armstrong, and B.J. Marshall. 1986. *Campylobacter pyloridis*, gastritis and peptic ulceration. *J. Clin. Pathol.* **39**: 353-365.

Goodwin, C.S., J.A. Armstrong, T. Chilvers, M. Peters, D. Collins, L. Sly, W. McConnel, and W.S. Harper. 1989. Transfer of *Campylobacter pylori* and Campylobacter mustelae to Helicobacter gen. Nov. as *Helicobacter pylori* comb. nov. and Helicobacter mustelae comb. nov., respectively. *Int. J. Syst. Bacteriol.* **4**: 397-405.

Goodwin, C.S., R.K. McCulloch, J.A. Armstrong, and S.H. Wee. 1985. Unusual cellular fatty acids and distinctive ultrastructure in a new spiral bacterium (*Campylobacter pyloridis*) from the human gastric mucosa. *J. Med. Microbiol.* **19**: 257-267.

Gordon, T., W.B. Kennel, W.P. Castelli, and T.R. Dawber, 1981. Lipoproteins, Cardiovascular disease, and death: the Franigham study. *Arch. Intern. Med.* **141**: 1128-1131.

Graham, D.Y. 1992. Pathogenic mechanisms leading to *Helicobacter pylori*- induced inflammation. *Eur. J. Gastroenterol. Hepatol.* **4** (Suppl. 2): 6-16.

Graham, D.Y. 1995. Clarithromycin for treatment of *Helicobacter pylori* infections. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1): S55-S58.

Graham, D.Y. 1997. *Helicobacter pylori* infection in the pathogenesis of duodenal ulcer and gastric Cancer: a model. *Gastroenterol.* **113**: 1983-1991.

Graham, D.Y. 2000. Therapy of *Helicobacter pylori*: Current status and issues. *Gastroenterol.* **118**: S2-S8.

Graham, D.Y., and W.A. Qureshi. 2001. Markers of infection. p. 499-510. In *Helicobacter pylori*: physiology and geneticseds.H.L.T. Mobley, G.L. Mendz, and S.L. Hazell. ASM Press. Washington DC.Feinleib, M. 1983. Review of the epidemiological evidences for a possible relationship between hypocholesterolemia and Cancer. *Cancer Res.* **40**: 2503-2507.

Graham, D.Y., A. Opekun, G.M. Lew, D.J. Evans, Jr., P.D. Klein, and D.G. Evans. 1990. Ablation of exaggerated meal-stimulated gastrin release in duodenal ulcer patients after clearance of *Helicobacter (Campylobacter) pylori* infection. *Am. J. Gastroenterol.* **85**: 394-398.

Graham, D.Y., and M.S. Osato. 2000. *H. pylori* in the pathogenesis of duodenal ulcer: Interaction between duodenal acid load, bile, and *H. pylori*. *Am. J. Gastroenterol.* **95**: 87-91.

Graham, D.Y., E. Adam, G.T. Reddy, J.P. Agarwal, R. Agarwal, D.G. Evans, H.M. Malaty, and D.G. Evans. 1991. Seroepidemiology of *Helicobacter pylori* infection in India. Comparison of developing and developed countries. *Dig. Dis. Sci.* **36**: 1084-1088.

Granoff, D.M., K.E. Sheetz, M.H. Nahm, J.V. Madassery, and P.G. Shackelford. 1988. Further immunologic evaluation of children who develop Haemophilus disease despite previous vaccination with type b polysaccharide vaccine. *Monogr. Allerg.* **23**: 256-268.

Green, S.J. and Nacy, C.A. 1993. Antimicrobial and immunopathological effects of cytokine induced nitric oxide synthesis. *Curr. Opin. Infect. Dis.* **6**: 384-396.

Grey, H.H., and H.G. Kunkel. 1964. H-chain subgroups of myeloma proteins and normal 7S-globulins. *J. Exp. Med.* **120**: 253-257.

Grisham, M.B., D. Jaurd'heuil, and D.A. Wink. 2000. Chronic inflammation and reactive oxygen and nitrogen metabolism- implications in DNA damage and mutagenesis. *Ailment. Pharmacol. Ther.* **14** (suppl. 1): 3-9.

Grubel, P. J.S. Hoffman, F.K. Chong, N.A. Burstein, C. Mepani, and D.R. Cave. 1997. Vector potential of houseflies (*Musca domestica*) for *Helicobacter pylori*. *J. Clin. Microbiol.* **35**: 1300-1303.

Guarner, J. A. Mhar, J. Parsonnet, and D. Halperin. 1993. The association of *Helicobacter pylori* with gastric Cancer and preneoplastic gastric lesions in Chiapas, Mexico. *Cancer* **71**: 297-301.

Guarner, J., J. Bartlett, D.P. Smith, M. Owens, R. Kreh, S. Czinn, and B.D. Gold. 2003. Can pre-neoplastic lesions be detected in gastric biopsies of children with *Helicobacter pylori* infection? *J. Paediatr. Gastroenterol. Nutr.* **37**: 309-314.

Guineay, D.G., P. Hasegawa, and S.P. Cole. 2003. *Helicobacter pylori* preferentially induces interleukin-12 (IL-12) rather than IL-6 or IL-10 in human dendritic cells. *Infect. Immun.* **71**: 4163-4166.

Gwynn, C.M., J. Morrisonsmith, G. Leonleon, and D.R. Stanworth. 1978. Role of IgG_4 subclass in childhood allergy. *Lancet* **1**: 910-911.

Haeberle, H. M. Kubin, K.B. Bamford, R. Garofalo, D.Y. Graham, F. El-Zaateri, R. Karttunen, S.E. Crowe, V.E. Reyes, and P.B. Ernst. 1997. Induction of IL-12 and selection of Th1 cells in gastric mucosa in response to H. pylori. Infect. Immun. **65**: 4229-4239.

Hamasaki, N., E.I.K. Tominaga, Y. Tezuka, T. Nagaoka, S. Kadota, T. Kuroki, and I. Yano. 2000. Highly selective antibacterial activity of novel alkyl quinolone alkaloids from Chinese herbal medicine, Gosyuyu (Wu-Chu-Yu), against *Helicobacter pylori in vitro. Microbiol. Immunol.* **44**: 9-15

Hamazoe, R., M. Maeta, T. Matsui, S. Shibata, S. Shiota, and N. Kaibara. 1992. CA72-4 compared with carcinoembryonic antigen as a tumour marker for gastric Cancer. *Eur. J. Cancer* **28A**: 1351-1354.

Hamilton, R.G. 1987. Human IgG subclass measurements in the clinical laboratory. *Clin. Chem.* **33**: 1707-1725.

Hammarstrom, L and C.I.E. Smith. 1983. IgG_2 deficiency in a healthy blood donor. Concomitant lack of IgG_2, IgA and IgE immunoglobulins and specific anti-carbohydrate antibodies. *Clin. Exp. Immunol.* **51**: 600-604.

Hammarstrom, L. and C.I.E. Smith. 1986. IgG subclasses in bacterial infections. *Monogr. Allergy* **19**: 122-133.

Hammarstrom, L., M. Granstrom, V. Oxelius, M.A.A. Persson and C.I.E. Smith. 1984. IgG subclass distribution of antibodies against *S. aureus* teichoic acid and α-toxin in normal and immunodeficient donors. *Clin. Exp. Immunol.* **55**: 593-601.

Hansson, L.R., L. Engstrand, O. Nyren, and L. Lindgren. 1995. Prevalence of *Helicobacter pylori* infection in subtype of gastric Cancer. *Gastroenterol.* **109**: 885-888.

Hara, K., Y. Morita, H. Kamihata, T. Iwasaka, and H. Takahashi. 2001. Evidence for infection with *Helicobacter pylori* in patients with acute myocardial infarction. *Clin. Chim. Acta.* **313**: 87-94.

Harats, D., M. Ben-Naim, Y. Dabach, G. Hollander, E. Havivi, O. Stein and Y. Stein. 1990. Effect of vitamin C and E supplementation on susceptibility of plasma lipoproteins to peroxidation induced by acute smoking. *Atherosclerosis* **85**: 47-54.

Harris, A.W., A. Douds, E.V. Meurisse, M. Dennis, S. Chambers and S.R. Gould. 1995. Seroprevalence of *Helicobacter pylori* in residents of a hospital for people with severe learning difficulties. *Eur. J. Gastroenterol. Hepatol.* **7**: 21-23.

Hartz, R.A., G. Meimarakis, E. Bayerdorffer, M. Stolte, T. Kirchner, and G. Enders. 1996. Characterization of lymphocytic infiltrates in *Helicobacter pylori*-associated gastritis. *Scand. J. Gastroenterol.* **31**: 222-228.

Hayashi, S., T. Sugiyama, K. Yokota, H. Isogai, E. Isogai, H. Shimomura, K. Oguma, M. Asaka, and Y. Hirai. 2000. Combined effect of rabamipide and ecabet sodium on *Helicobacter pylori* adhesion to gastric epithelial cells. *Microbiol. Immunol.* **44**: 557-562.

Hazell, S.L., A. Lee, L. Brady, and W. Hennessy. 1986. *Campylobacter pyloridis* and gastritis: association with intracellular spaces and adaptation to an environment of mucus as important factors in colonization of gastric epithelium. *J. Infect. Dis.* **153**: 658-663.

Hazzell, S.L., D.J. Evans, Jr. and D.Y. Graham. 1991. *Helicobacter pylori* catalase. *J. Gen. Microbiol.* **137**: 57-61.

Heep, M., S. Odenbreit, D. Beck, J. Decker, E. Prohaska, U. Reiger, and N. Lehn. 2000. Mutations at four distinct regions of the rpoB gene can reduce susceptibility of *Helicobacter pylori* to rifamycins. *Antimicrob. Agents Chemother.* **44**: 1713-1715.

Heinicke, J.W., H. Rosen, and A. Chait. 1987. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest.* **74**: 1890-1894.

Henriksen, T.H., O. Brorson, S. Schoyen, T. Thoresen, and A. Lia. 1997. A simple method for determining metronidazole resistance of *Helicobacter pylori*. *J. Clin. Microbiol.* **35**: 1424-1426.

Hirai, M., T. Azuma, S. Ito, T. Kato, Y. Kohli, and N. Fujiki. 1994. High prevalence of neutralizing activity to *Helicobacter pylori* cytotoxin in serum of gastric carcinoma patients. *Int. J. Cancer* **56**: 56-60.

Hirayama, F., S. Takagi, Y. Yokogama, E. Iwao, and Y. Ikeda. 1996. Establishment of gastric *Helicobacter pylori* infection in Mongolian gerbils. *J. Gastroenterol.* **31**: 24-28.

Hirschl, A.M., M. Pletschette, M.H. Hirschl, J. Berger, G. Stanek, and M.L. Rotter. 1988. Comparison of different antigen preparations in an evaluation of the immune response to *Campylobacter pylori*. *Eur. J. Clin. Microbiol. Infect. Dis.* **7**: 570-575.

Ho, B., and B. Jiang. 1995. The adhesion of *Helicobacter pylori* extract to four mammalian cell lines. *Eur. J. Gastroenterol. Hepatol.* **7**: 121-124.

Hong, W., S. Murimatsu, T. Goto, G. Sachs, D.R. Scott, D.L. Weeks, T. Kohno, C. Morita, T. Nakano, Y. Fujioka, and K. Sano. 2000. Contrast enhanced immunoelectron microscopy for *Helicobacter pylori*. *J. Microbiological Methods* **42**: 121-127.

Hopkins, R.J., P.A.Vial, C. Ferreccio, J. Ovalle, P. Prado, V. Sotomayor, R.G. Russell, S.S. Wasserman, and J.G. Morris, Jr. 1993. Seroprevalence of *Helicobacter pylori* in Chile: vegetables may serve as one route of transmission. *J. Infect. Dis.* **168**: 222-226.

Hu, L.T., P.A. Foxall, R. Russell and H.L.T. Mobley. 1992. Purification of recombinant *Helicobacter pylori* urease apoenzyme encoded by urea and ureB. *Infect. Immun.* **60**: 2657-2666.

Huang, J., P.W.N. Keeling, and C.J. Smyth. 1992. Identification of erythrocyte-binding antigens in *Helicobacter pylori*. *J. General Microbiol.* **138**: 1503-1513.

Hurtado, A., B. Chahal, R.J. Owen, and A.W. Smith. 1994. Genetic diversity of *Helicobacter pylori* haemaglutinin/protease (hap) gene. *FEMS Microbiol. Lett.* **123**: 173-178.

Hussel, S., P.G.Isaacson, J. E. Crabtree, and J. Spencer. 1993. The response of cells from low-grade B-cell gastric lymphomas of mucosa associated lymphoid tissue to *Helicobacter pylori*. *Lancet* **342**: 571-574.

Hwang, H., J. Dwyer, and R.M. Russell. 1994. Diet, *Helicobacter pylori* infection, food preservation and gastric cancer risk: are there new roles for preventative factors? *Nutr. Rev.* **52**: 75-83.

Hynes, S.O., N. Broutet, Eurohepygast study group, T. Wadstrom, M. Mikelsaar, P.W. O'Toole, J. Telford, L. Engstrand, S. Kamiya, A.F. Mentis, and A.P. Moran. 2002. Phenotypic variations of *Helicobacter pylori* isolates from geographically distinct regions detected by lectin typing. *J. Clin. Microbiol.* **40**: 227-232.

Ibrahimov, A., and J. Pappo. 2000. The immune response against *Helicobacter pylori* - a direct linkage to the development of gastroduodenal disease. *Microbes and Infection* **2**: 1073-1077.

Igarashi, H., S. Takahashi, N. Ishiyama, K. Nakamura, N. Masubuchi, S. Saito, T. Aoyagi, T. Ito, and I: Hirata. 1992. Is *Helicobacter pylori* a causal agent in gastric carcinoma? *Irish. J. Med. Sci.* **161**: 69.

Ikewaki, J., A. Nishizono, T. Fujioka, and K. Mifune. 2000. Therapeutic oral vaccination induces mucosal immune response sufficient to eliminate long-term *Helicobacter pylori* infection. *Microbiol. Immunol.* **44**: 29-39.

Iskander, R., P.K. Das, and R.C. Aalberse. 1981. IgG_4 antibodies in Egyptian patients with schistosomiasis. *Int. Arch. Allergy Appl. Immunol.* **66**: 200-207.

Isogai, H., E. Isogai, K. Kimura, N. Fuji, K. Yokota, and K. Oguma. 1994. *Helicobacter pylori* induces inflammation in mouse urinary bladder and pelvis. *Microbiol. Immunol.* **38**: 331-336.

Jablonowski, H., K.J. Hengels, N. Kramer, G. Geis, W. Opferkuch, and G. Strohmeyer. 1994. Effect of *Helicobacter pylori* on dbc-AMP stimulated acid secretion by human parietal cells. *Hepatogastroenterol.* **41**: 546-548.

Jarbrink, Q.M., I. Ahlstedt, C. Lindholm, E.L. Johansson, and H. Lonroth. 2001. Homing commitment of lymphocytes activated in the human gastric and intestinal mucosa. *Gut* **49**: 519-525.

Jaskowski, T.D., T.B. Martins, H.R. Hill, and C.M. Litwin. 1997. Immunoglobulin A antibodies to *Helicobacter pylori*. *J. Clin. Microbiol.* **35**: 2999-3000.

Ji, X., M. Shen, P. Jia, and X. Long. 1993. *Helicobacter pylori* associated gastritis and gastric carcinoma in Beijing. *J. Environ. Pathol. Toxicol. Oncol.* **12**: 209-212.

Jobin, C. and R.B. Sartor. 2000. The I kappa B/NF-kappa B system: a key determinant of mucosal inflammation and protection. *Am. J. Physiol. Cell Physiol.* **278**: C451.

Johansen, H.K., A. Norgaard, L.P. Andersen, P. Jensen, H. Nielsen, and N. Hoiby. 1995. Cross reactive antigens shared by *Pseudomonas aeruginosa*, *Helicobacter pylori*, Campylobacter jejuni, and *Haemophilus influenzae* may cause false positive titres of antibody to *H. pylori*. *Clin. Diagn. Lab. Immunol.* **2**: 149-155.

Jones, D.M., A. Curry, and A.J. Fox. 1985. An ultrastructural study of the gastric Campylobacter-like organism "*Campylobacter pyloridis*". *J. Gen. Microbiol.* **131**: 2335-2341.

Josenhans, C., K.A. Eaton, T. Thevenot, and S. Suerbaum. 2000. Switching of flagellar motility in *Helicobacter pylori* by reversible length variation of a short homopolymeric sequence repeat in flip, a gene encoding a basal body protein. *Infect. Immun.* **68**: 4598-4603.

Kamisago, S., M. Iwamori, T. Tai, K. Mitamura, Y. Yazaki, and K. Sugano. 1996. Role of sulfatides in adhesion of *Helicobacter pylori* to gastric Cancer cells. *Infect. Immun.* **64**: 624-628.

Karita, M., M. Tsuda, K. Okita, T. Sugiyama, and T. Nakazawa. 1995. Evaluation of a new bismuth-free triple therapy in nude mice and humans. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1: S31-S34.

Karttunen, R., G. Andersson, K. Poikonen, T.U. Kousunen, T. Karttunen, K. Juutinen and S. Niemela. 1990. *Helicobacter pylori* induces lymphocyte activation in peripheral blood cultures. *Clin. Exp. Immunol.* **82**: 485-488.

Karttunen, R., T. Karttunen, H.-P.T. Ekre, and T.T. MacDonald. 1995. Interferon gamma and interleukin-4 secreting cells in the gastric antrum in *Helicobacter pylori* positive and negative gastritis. *Gut* **36**: 341-345.

Karvar, S., H. Karch, M. Frosch, W. Burghardt and U. Gross. 1997. The use of specific immunoglobulin A and G for detection of *Helicobacter pylori* infection in patients with chronic gastritis by immunoblot analysis. *J. Clin. Microbiol.* **35**: 3058-3061.

Katelaris, P.H., and D.B. Jones. 1997. Testing for *Helicobacter pylori* infection after antibiotic treatment (editorials) *Am. J. Gastroenterol.* **92**: 1245-1247.

Katelaris, P.H., G.H.K. Tippet, P. Norbu, D.G. Lowe, R. Brennan, and M.J.G. Farthing. 1992. Dyspepsia, *Helicobacter pylori* and peptic ulcer in a randomly selected population in India. *Gut* **33**: 1462-1466.

Kawai, T., M. Seki, K. Hiromatsu, J.W. Eastcott, G.F.M. Watts, M. Sugai, D.J. Smith, S.A. Porcelli, and M.A. Taubman. 1999. Selective diapedisis of Th1 cells induced by endothelial cell RANTES. *J. Immunol.* **163**: 3269-3278.

Kawanishi, M., S. Fukuda, H. Kawaguchi, K. Kohmoto, K. Haruma, and G. Kajiyama. 1995. Significance of rapid urease test for identification of *Helicobacter pylori* in comparison with hisological and culture studies. *J. Gastroenterol.* **30**: 16-20.

Keats, S., Y.S. Hitti, M. Upton, and C.P. Kelley. 1997. *Helicobacter pylori* infection activates NF-κB in gastric epithelial cells. *Gastroenterol.* **113**: 1099-1109.

Keenan, J., J. Oliaro, N. Domigan, H. Potter, G. Aitken, R. Allardyce, and J. Roake. 2000. Immune response to an 18-kilodalton outer membrane antigen identifies Lipoprotein 20 as a vaccine candidate. *Infect. Immun.* **68**: 3337-3343.

Khanna, A.K., P. Seth, G. Nath, V.K. Dixit, and M. Kumar. 2002. Correlation of *Helicobacter pylori* and gastric carcinoma. *J. Postgrad. Med.* **48**: 27-28.

Kikuchi, S., M. Kurosawa, T. Sakiyama, H. Tenjin, K. Miki, O. Wada, and Y. Inaba. 2000. Long-term effect of *Helicobacter pylori* infection on serum pepsinogens. *Jpn. J. Cancer Res.* **91**: 471-476.

Kim, J., H. Jung, J.M. Kim, I. Song, and C. Kim. 1998. Interleukin-8 expression by human neutrophils activated by *Helicobacter pylori* soluble proteins. *Scand. J. Gastroenterol.* **33**: 1249-1255.

Kim, J.J., H. Tao, E. Carloni, W.K. Leung, D.Y. Graham, and A.R. Sepulveda. 2002. *Helicobacter pylori* impairs DNA mismatch repair in gastric epithelial cells. *Gastroenterol.* **123**: 542-553.

Kim, J.M., J.S. Kim, H.C. Jung, I.S. Song, and C.Y. Kim. 2000. Apoptosis of human gastric epithelial cells via caspase 3 activation in response to *Helicobacter pylori* infection: possible involvement of neutrophils through necrosis factor alpha and soluble Fas ligands. *Scand. J. Gastroenterol.* **35**: 40-48.

Kimmel, B., A. Bosserhoff, R. Frank, R. Gross, W. Goebel, and D. Beier. 2000. Identification of immunodominant antigens from *Helicobacter pylori* and evaluation of their reactivities with sera from patients with different gastroduodenal pathologies. *Infect. Immune.* **68**: 915-920.

Kindermann, A., N. Konstandopoulos, N. Lehn, H. Demmelmair, and S. Koletzko. 2001. Evaluation of two commercial enzyme immunoassays, testing immunoglobulin G (IgG) and IgA responses, for diagnosis of *Helicobacter pylori* infection in children. *J. Clin. Microbiol.* **39**: 3591-3596.

Kitamoto, N., H. Nakamoto, A. Katai, N. Takahara, H. Nakata, H. Tamaki, and T. Tanaka. 1998. Heterogeneity of protein profiles of *Helicobacter pylori* isolated from individual patients. *Helicobacter* **3**: 152-162.

Klaamas, K., M. Held, T. Wadstrom, A. Lipping, and O. Kurtenkov. 1996. IgG immune response to *Helicobacter pylori* antigens in patients with gastric *Cancer* as defined by ELISA and immunoblotting. *Int. J. Cancer* **67**: 1-5.

Klausz, G., A. Tiszai, L. Tiszlavicz, Z. Gyulai, Z. Lenart, J. Lonovics, and Y. Mandi. 2003. Local and peripheral cytokine response and cagA status of *Helicobacter pylori* positive patients with duodenal ulcer. *Eur. Cytokine Netw.* **14**: 143-148.

Klebanoff, S.J. 1998. Phagocytic cells: products of oxygen metabolism. p. 391-445. In Inflammation: basic principles and clinical correlates. Ed J.I. Gallin, I.M. Goldstein, and R. Snyderman. Raven Press, NewYork.

Klein, P.D. and Graham, D.Y. 1993. Minimum analysis requirements for the detection of *Helicobacter pylori* infection by the ^{13}C-urea breath test. *Am. J. Gastroenterol.* **88**: 1865-1869.

Klein, P.D., H.M. Malaty, R.F. Martin, K.S. Graham, R.M. Genta, and D.Y. Graham. 1996. Noninvasive detection of *Helicobacter pylori* infection in clinical practice: ^{13}C–urea breath test. *Am. J. Gastroenterol.* **91**: 690-694.

Klein, P.D., R.H. Gilman, R. Leonbarua, F. Diaz, E.O. Smith, and D.Y. Graham. 1994. The epidemiology of *Helicobacter pylori* in Peruvian children between 6 and 30 months of age. *Am. J. Gastroenterol.* **89**: 2196-2200.

Knipp, U., S. Birkholz, W. Kaup, and W. Opperkuch. 1996. Partial characterization of a cell proliferation-inhibiting protein produced by *Helicobacter pylori*. *Infect. Immun.* **64**: 3491-3496.

Kobayashi, D., Y. Eishi, T. Ohkusa, I. Ishige, T. Suzuki, J. Minami, T. Yamada, T. Takizawa, and M. Koike. 2002. Gastric mucosal density of *Helicobacter pylori* estimated by real-time PCR compared with results of urea breath test and histological grading. *J. Med. Microbiol.* **51**: 305-311.

Kokkola, A., H. Rautelin, P. Puolakkainen, P. Sipponen, M. Farkkila, R. Haapiainen, and T.U. Kousunen. 2000. Diagnosis of *Helicobacter pylori* infection in patients with atrophic gastritis: Comparison of histology, 13C-urea breath test, and serology. *Scand. J. Gastroenterol.* **35**: 138-141.

Koster, T., and J.P. Vanderbroucke. 1992. *Helicobacter pylori*, musings from the epidemiological armchair. *Epidemiol. Infect.* **109**: 81-85.

Kostrzynska, M., J.D. Betts, J.W. Austin, and T.J. Trust. 1991. Identification, characterization, and spatial localization of two flagellin species in *Helicobacter pylori* flagella. *J. Bacteriol.* **173**: 937-946.

Krajden, S., M. Fuksa, J. Anderson, J. Kempston, A. Boccia, C. Petrea, C. Babida, M. Karmali, and J.L. Penner. 1989. Examination of human stomach biopsies, saliva and dental plaque for *Campylobacter pylori*. *J. Clin. Microbiol.* **27**: 1397-1398.

Kubes, P. and D.M. McCafferty. 2000. Nitric oxide and intestinal inflammation. *Am. J. Med.* **109**: 150-153.

Kuipers, E.J. 1998. Relationship between *Helicobacter pylori*, atrophic gastritis and gastric cancer. *Ailment. Pharmacol. Ther.* **12**: 25-36.

Kuipers, E.J., A.M. Uyterlinde, A.S. Pena, R. Roosendaal, G. Pals, G.F. Nelis, H.P.M. Festen, and S.G.M. Meuwissen. 1995. Long-term sequelae of *Helicobacter pylori* gastritis. *Lancet* **345**: 1525-1528.

Kuipers, E.J., D.A. Israel, J.G. Kusters, M.M. Gerrits, J. Weel, A. van der Ende, R.W.M. van der Hulst, H.P. Wirth, J.H. Nikanne, S.A. Thompson, and M.J. Blaser. 2000. Quasi species development of *Helicobacter pylori* observed in paired isolates obtained years apart from the same host. *J. Infect. Dis.* **181**: 273-282.

Kuipers, E.J., M.G. Cassanova, A.S. Pena, G. Pals, G. van Kamp, A. Kok, E. K. Pohlmann, N.F.M. Pels, and S.G.M. Meuwissen. 1993. *Helicobacter pylori* serology in patients with gastric carcinoma. *Scand. J. Gastroenterol.* **28**: 43-437.

Kumar, D., A. Dhar, S. Dattagupta. V. Ahuja, M. Mathur, and M.P. Sharma. 2002. Pre and post eradication gastric inflammation in *Helicobacter pylori*-associated duodenal ulcer. *Indian J. Gastroenterol.* **21**: 7-10.

Kumar, D., and A.N. Sinha, 2002. *Helicobacter pylori* infection delays ulcer healing in patients operated for perforated duodenal ulcer. *Indian J. Gastroenterol.* **21**: 19-22.

Kumar, D., C.S. Bal, S. Dattagupta, V. Ahuja, M. Mathur, and M.P. Sharma. 2001. ^{14}C urea breath test does not predict density of *Helicobacter pylori* in duodenal ulcer disease. *Indian J. Med. Res.* **113**: 129-134.

Kumar, S., A. Dhar, S. Srinivasan, S. Jain, A. Rattan, and M.P. Sharma. 1998. Antibodies to CagA protein are not predictive of serious gastroduodenal disease in Indian patients. *Indian J. Gastroenterol.* **17**: 126-128.

Kusama, K., T. Sekine, M. Adachi, H. Ohgaki, T. Hirota, S. Morinaga, H. Hasegawa, N. Hayasaka, I. Moro, and S. Sato. 1987. Derivation of a murine monoclonal antibody useful for immunohistochemical diagnosis of human adenoid cystic carcinoma of stomach *Cancer. Jpn. J. Cancer Res.* **78**: 952-959.

Kutukculer, N., S. Aydogdu, S. Caglayan, and R.V. Yogei. 1998. Salivary and gastric fluid secretory immunoglobulin A and free secretory component concentration in children with *Helicobacter pylori* positive gastritis. *J. Trop. Paediatr.* **44**: 178-180.

Kwon, D.H., F.A.K. El-Zaatari, M. Kato, M.S. Osato, R. Reddy, Y. Yamaoka, and D.Y. Graham. 2000. Analysis of rdxA and involvement of additional genes encoding NAD(P)H flavin oxidoreductase (FrxA) and ferredoxin-like protein (FdxB) in metronidazole resistance of *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **44**: 2138-2142.

Labigne, A., V. Cussac, and P. Courcoux. 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. *J. Bacteriol.* **173**: 1920-1931.

Lachman, L., and C.W. Howden. 2000. Twenty four hour intragastric pH: tolerance within 5 days of continuous ranitidine administration. *Am. J. Gastroenterol.* **95**: 57-61.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* **227**: 680-685.

Langenberg, M.L., G.N.J. Tytgat, M.E.I. Schipper, P.J.G.M. Reitra, and H.C. Zanen. 1984. Campylobacter-like organisms in the stomach of patients and healthy individuals. *Lancet* **i**: 1348.

Laurila, A., A. Bloigu, S. Nayha, J. Hassi, M. Leinonen, and P. Saikku. 1999. Association of *Helicobacter pylori* with elevated serum lipids. *Atherosclerosis* **142**: 207-210.

LaVecchia, C., E. Negri, S. Franceschi, and A. Gantile. 1992. Family history and the risk of stomach and colorectal cancer. *Cancer* **70**: 50-55.

Leaf, C.D., J.S. Wisknok, and S.R. Tannenbaum. 1991. Endogenous incorporation of nitrogen oxide from L-arginine into N-nitrosomorphine stimulated *Escherichia coli* lipopolysaccharide in the rat. *Carcinogenesis* **12**: 537-539.

Lehman, F.S., and G.A. Stadler. 1998. Hypothesis on the role of cytokines in peptic ulcer disease. *Eur. J. Clin. Invest.* **28**: 511-519.

Leung, W.K., J.J. Kim, J.G. Kim, D.Y. Graham, and A.R. Sepuveda. 2000. Microsatallite instability in gastric intestinal metaplasia in patients with and without gastric Cancer. *Am. J. Pathol.* **156**: 537-543.

Leunk, R.D., P.T. Johnson, B.C. David, W.G. Kraft, and D.R. Morgan. 1988. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *J. Med. Microbiol.* **26**: 93-99.

Levi, S., C.T. Dollery, S.R. Bloom, J. Calam, F.M. Cox, H.J. Hodgdon, M.S. Losowsky, M.B. Pepys, N.A. Wright, and O.M. Wrong. 1989. *Campylobacter pylori*, duodenal ulcer disease, and gastrin. *Br. Med. J.* **299**: 1093-1094.

Li, H., C. Stoicov, X. Cai, T.C. Wang, and J. Houghton. 2003. *Helicobacter* and gastric cancer disease mechanisms: host response and disease susceptibility. *Curr. Gastroenterol. Rep.* **5**: 459-467.

Lichtenberger, L.M., and J.J. Romero. 1994. Effect of ammonium ion on the hydrophobic and barrier properties of the gastric mucus gel layer: Implications on the role of ammonium in *H. pylori*-induced gastritis. *J. Gastroenterol. Hepatol.* **9**: S13-S19.

Lin, J.T., J.T. Wang, M.S. Wu, T.S. Huang, S.W. How, H.P. Wang, T.M. Chan, and T.H. Wang. 1994. Serological, histological, and polymerase chain reaction studies of *Helicobacter pylori* infection in patients with gastric adenocarcinoma. *J. Formos. Med. Assoc.* **93**: 15-19.

Lin, J.T., J.T. Wang, T.H. Wang, M.S. Wu, T.K.Lee, and C.J. Chen. 1993. *Helicobacter pylori* infection in a randomly selected population, Healthy volunteers, and patients with gastric ulcer and gastric adenocarcinoma: a seroprevalence study in Taiwan. *Scand. J. Gastroenterol.* **28**: 1067-1072.

Lin, S.K., J.R. Lambert, M. Schembri, L. Nicholson, M. Finaly, C. Wong, and A. Coulepis. 1992. A comparison of diagnostic tests to determine *Helicobacter pylori* infection. *J. Gastroenterol. Hepatol.* **7**: 203-209.

Lingwood, C.A., H. Law, A. Pellizari, P. Sherman, and B. Drumm. 1989. Gastric glycerolipid as a receptor for Campylobacter pylori. *Lancet* **I**: 238-241.

Lipid research clinic programme. 1984. The lipid research clinics coronary disease primary prevention trials results I and II: Reduction in incidence of coronary heart disease to cholesterol lowering. *JAMA* **251**: 351-374.

Liutu, M., K. Kalimo, H. Kalimo, J. Uksila, and R. Leino. 2004. Mast cell and IgE containing cells in gastric mucosa of *Helicobacter pylori* infected and non-infected patients with chronic urticaria. *J. Eur. Acad. Dermatol. Venereol.* **18**: 69-72.

Logan, R.P.H., A. Robins, G.A. Turner, A. Cockayne, S.P. Borriello, and C.J. Hawkey. 1998. A novel flow cytometric assay for quantitating adherence of *Helicobacter pylori* to gastric epithelial cells. *J. Immunol. Methods* **213**: 19-30.

Loivukene, K., H. Kolk, H.I. Maaroos, P. Kasenomm, H. Aro, M. Ustav, and M. Mikelsaar. 2000. Metronidazole and Clarithromycin susceptibility and the subtypes of VacA of *Helicobacter pylori* isolates in Estonia. *Scand. J. Infect. Dis.* **32**: 59-62.

Luzza, F., G. Oderda, M. Maletta, M. Imeneo, L. Mesuraca, E. Chioboli, P. Lerro, S. Guandalini, and F. Pallone. 1997. Salivary immunoglobulin G assay to diagnose *Helicobacter pylori* infection in children. *J. Clin. Microbiol.* **35**: 3358-3360.

Lynch, D.A.F., and A.T.R.Axon. 1995. *Helicobacter pylori*, gastric *Cancer* and gastric epithelial kinetics. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1): S17-S23.

Lynch, D.A.F., N.P. Mapstone, A.M.T. Clarke, G.M. Sobala, P. Jackson, L. Morrison, M.F. Dixon, P. Quirke, and A.T.R. Axon. 1995a. Cell proliferation in *Helicobacter pylori* associated gastritis and the effect of eradication therapy. *Gut* **36**: 346-350.

Lynch, D.A.F., N.P. Mapstone, A.M.T. Clarke, P. Jackson, M.F. Dixon, P. Quirke, and A.T.R. Axon. 1995b. Cell proliferation in the gastric corpus in *Helicobacter pylori* associatd gastritis and after resection. *Gut* **36**: 351-353.

Maaroos, H.I., M. Kekki, T. Vorobjova, V. Salupere, and P. Sipponen. 1994. Risk of recurrence of gastric ulcer, chronic gastritis, and grade of *Helicobacter pylori* colonization: a long-term follow-up study of 25 patients. *Scand. J. Gastroenterol.* **29**: 532-536.

Mac Dermott, R.P., G.S. Nash, I.O. Auer, R. Shlein, B.S. Lewis, J. Madassery, and M.H. Nahm. 1989. Alterations in serum immunoglobulin G subclasses in patients with ulcerative colitis and Crohn's disease. *Gastroenterol.* **96**: 764-768.

Maeda, S., H. Yoshida, H. Matsunaga, K. Ogura, O. Kawamata, Y. Shiratori, and M. Omata. 2000. Detection of clarithromycin resistant *Helicobacter pylori* strains by a preferential homoduplex formation assay. *J. Clin. Microbiol.* **38**: 210-214.

Maeda, S., H. Yoshida, Y. Mitsuno, Y. Hirata, K. Ogura, Y. Shiratori, and M. Omata. 2002. Analysis of apoptotic and antiapoptotic signaling pathways induced by *Helicobacter pylori*. *Gut* **50**: 771-778.

Magnusson, C.G.M., J.Y. Cesbron, R. Djurup, A. Capron, and S.G.O. Johansson. 1986. Raised serum IgG_4 levels in patients with atopy and filariasis: application of an automated particle-counting immunoassay using monoclonal antibody. *Int. Arch. Allergy Appl. Immunol.* **81**: 238-244.

Mai, U.E.H., G.I. Perez-Perez, L.M. Wahl, S.M. Wahl, M.J. Blaser, and P.D. Smith. 1991. Soluble surface proteins from *Helicobacter pylori* activate monocyte/macrophages by lipopolysaccharide independent mechanism. *J. Clin. Invest.* **87**: 894-900.

Majumdhar, P.S.M. Shah, K.R. Dhunjibhoy, and H.G. Desai. 1990. Isolation of Helicobcater pylori from dental plaques in healthy volunteers. *Indian J. Gastroenterol.* **9**: 271-272.

Malaty, H.M., A. El-Kasabany, D.Y. Graham, C.C. Miller, S.G. Reddy, S.R. Srinivasan, Y. Yamaoka, and G.S. Berenson. 2002. Age at acquisition of *Helicobacter pylori* infection: a follow-up study from infancy to adulthood. *Lancet* **359**: 931-935.

Malaty, H.M., D.Y. Graham, W.A. Wattigney, S.R. Srinivasan, M. Osato, and G.S. Berenson. 1999. Natural history of *Helicobacter pylori* infection in childhood: 12-year follow-up cohort study in biracial community. *Clin. Infect. Dis.* **28**: 279-282.

Manetti, R., P. Massari, M. Marchetti, C. Magagnoli, S. Nuti, P. Lupetti, P. Ghiara, R. Rappuoli, and J.L. Telford. *Infect. Immun.* **65**: 4615-4619.

Manoharan, S., K. Kavitha, and S. Nagini. 1997. Role of life-style on plasma and erythrocyte membrane lipid profile in gastric *Cancer* patients. *Indian J. Physiol. Pharmacol.* **41**: 62-66.

Marchetti, M., B. Arico, D. Burroni, N. Figura, R. Rappuoli, and P. Ghiara. 1995. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science* **267**: 1655-1658.

Marshall, B.J., and J.R. Warren 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **i**:1311-1315

Marshall, B.J., and S.R. Langton. 1986. Urea hydrolysis in patients with *Campylobacter pyloridis* infection. *Lancet* **i**: 865-866.

Marshall, B.J., J.A. Armstrong, D.B. McGechie, and R.J. Glancy. 1985. Attempt to fulfil Koch's postulates for pyloric Campylobacter. *Med. J. Aus.* **142**: 436-439.

Marshall, B.J., L.J. Barrett, C. Prakash, R.W. McCallum, and R.L. Guerrant. 1990. Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bactericidal effect of acid. *Gastroenterol.* **99**: 697-702.

Matsubara, S., H. Shibata, F. Ishikawa, T. Yokokura, M. Takahashi, T. Sugimura, and K. Wakabayashi. 2003. Suppression of Helicobacter pylori–induced gastritis by green tea extract in Mongolian gerbils. *Biochem. Biophys. Res. Commun.* **310**: 715-719.

Matsui, T., Y. Matsukawa, T. Sakai, K. Nakamura, A. Aoike, and K. Kawai. 1995. Effect of ammonia on cell-cycle progression of human gastric *Cancer* cells. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1): S79-S81.

Matsukura, N., M. Onda, A. Tokunaga, N. Matsuda, and K. Yamashida. 1995. Mucosal IgA antibody against *Helicobacter pylori* in chronic gastritis and intestinal metaplasia detected by Tes-Tape method in resection specimens after gastrectomy for gastric Cancer. *Cancer* **75**: 1472-1477.

Mattsson, A., M.Q. Jarbrink, H. Lonroth, A. Hamlet, I. Ahlstedt, and A.M. Svennerholm. 1998. Antibody-secreting cells in the stomachs of symptomatic and asymptomatic *Helicobacter pylori*-infected subjects. *Infect. Immun.* **66**: 2705-2712.

Mayumi, M., T. Kuritani, H. Kubagawa, and M.D. Kooper. 1983. IgG subclass expression by human B lymphocytes precommitted to IgG subclass can be preferentially induced by polyclonal mitogens with T cell help. *J. Immunol.* **130**: 671-677.

Mayus, J., K. Macke, P. Shackelford, J. Kim, and M. Nahm. 1986. Human IgG subclass assays using a novel assay method. *J. immunological Methods* **88**: 65-73.

McNulty, C.A.M., J.C. Dent, J.S. Uff, M.W.L. Gear, and S.P. Wilkinson. 1989. Detection of *Campylobacter pylori* by the biopsy urease test: an assessment in 1445 patients. *Gut.* **30**: 1058-1062.

Megraud, F. 1995. Rationale for the choice of antibiotics for the eradication of *Helicobacter pylori*. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1) S49-S54.

Megraud, F., M.P.B. Rabbe, F. Denis, A. Belbouri, and D.Q. Hoa. 1989. Seroepidemiology of *Campylobacter pylori* infection in various populations. *J. Clin. Microbiol.* **27**: 1870-1873.

Megraud, F., R. Cayla, H. Lamouliatte, S. Bouchard, and V. Darmaillae. 1994. Surveillance of *Helicobacter pylori* resistance to macrolides and nitroimidazole compounds at a national level. *Am. J. Gastroenterol.* **89**: 1368.

Meijer, B.C., J.C. Tjijs, J.H. Kleibeuker, A.A. van Zwet, and R.J.P. Berrelkamp. 1997. Evaluation of eight enzyme immunoassays for detection of immunoglobulin G against *Helicobacter pylori*. *J. Clin. Microbiol.* **35**: 292-294.

Meltzer, M.S., and C.A. Nacy. 1989. Delayed type hypersensitivity and the induction of activated, cytotoxic macrophages. p. 765-777. In Fundamental immunology. ed. W.E. Paul. Raven Press. NewYork.

Mendall, M.A., and T.C. Northfield. 1995. Transmission of *Helicobacter pylori* infection. *Gut* **37**: 1-3.

Mendall, M.A., P.M. Goggin, N. Molineaux, J. Levy, T. Toosy, D. Strachan, and T.C. Northfield. 1992. Childhood living conditions and *Helicobacter pylori* seropositivity in adult life. *Lancet* **339**: 896-897.

Mendall, M.A., R.P. Jazrawi, J.M. Morrero, N. Molineaux, J. Levi, J.D. Maxwell, and T.C. Northfield. 1995. Serology for *Helicobacter pylori* compared with symptom questionnaires in screening before direct access endoscopy. *Gut* **36**: 330-333.

Menagatti, M., D. Vaira, J. Holton, F. Miranda, C. Ricci, R. Gusmaroli, C. Ainley, M. Miglioli, and L. Barbara. 1996. Serological response to *Helicobacter pylori* in gastric and non-gastric *Cancer. Clin. Sci.* **91**: 219-223

Mertz, H.R., W.L. Peterson, and J.H. Walsh. 2000. "Familial hyperpepsinogenemia" and *Helicobacter pylori* infection. *Am. J. Gastroenterol.* **95**: 943-946.

Midolo, P.D., B. Dakic, L. Nicholson, J.R. Lambert, S. Lin, and E.G. Russell. 2000. Use of a dry latex agglutination test to measure eradication of *Helicobacter pylori* infection. *J. Gastroenterol. Hepatol.* **15**: 254-256.

Midolo, P.D., J.R. Lambert, E.G. Russell, and S.K. Lin. 1995. A practical single sample dry latex agglutination test for *Helicobacter pylori* antibody detection. *J. Clin. Pathol.* **48**: 969-971.

Miehlke, S., C. Kirsch, K.A. Amiri, T. Gunther, N. Lehn, P. Malfertheiner, M. Stolte, G. Ehninger, and E. Bayerdorffer. 2000. The *Helicobacter pylori* vacA s1, m1 genotype and cagA is associated with gastric carcinoma in Germany. *Int. J. Cancer* **87**: 322-327.

Miglio, F., M. Miglioli, V. Mazzeo, J. Holton, P. Mule, M. Menegatti, P. Maiolo, E. Grazia, A. Bini, R. Gusmaroli, A. Casadei, D. Velpiani, M. Gaudio, and R. Bazzocchi. Prevalence of *Helicobacter pylori* infection (HP) in patients with gastric carcinoma (GC). *Irish. J. Med. Sci.* **161**: 70.

Milman, N. S. Rosenstock, L. Andersen, T. Jorgensen, and O. Bonnevie. 1998. Serum ferritin, haemoglobin, and *Helicobacter pylori* infection: a seroepidemiologic survey comprising 2794 Danish adults. *Gastroenterol.* **115**: 268-274.

Misra, V., S. Misra, M. Dwivedi, U.P.A. Singh, V. Bhargava, and S.C. Gupta. 2000. A topographic study of *Helicobacter pylori* density, distribution, and associated gastritis. *J. Gastroenterol. Hepatol.* **15**: 737-743.

Mitchell, H. M., S.L. Hazell, T. Kolesnikow, J. Mitchell, and D. Frommer. 1996. Antigen recognition during progression from acute to chronic infection with cagA-positive strain of *Helicobacter pylori*. *Infect. Immun.* **64**: 1166-1172.

Mitchell, H.M. 2001. Epidemiology of infection. p. 7-18. In *Helicobacter pylori*: Physiology and Genetics.eds.H.L.T. Mobley, G.L. Mendz, and S.L. Hazell. ASM Press. Wahington. D.C.

Mitchell, H.M., Y.Y. Li, P.J. Hu, Q. Liu, M. Chen, G.G. Du, Z.J. Wang, A. Lee, and S.L. Hazell. 1992. Epidemiology of *Helicobacter pylori* in Southern China: Identification of early childhood as the critical period for acquisition. *J. Infect. Dis.* **166**: 149-153.

Miyaji, H., T. Azuma, S. Ito, Y. Abe, F. Gejyo, N. Hashimoto, H. Sugimoto, H. Suto, Y. Ito, Y. Yamazaki, Y. Kohli, and M. Kuriyama. 2000. *Helicobacter pylori* infection occurs via close contact with infected individuals in early childhood. *J. Gastroenterol. Hepatol.* **15**: 257-262.

Mizuki, I., T. Shimoyama, S. Fukuda, Q. Liu, S. Nakaji, and A. Munakata. 2000. Association of gastric epithelial apoptosis with the ability of *Helicobacter pylori* to induce neutrophil oxidative burst. *J. Med. Microbiol.* **49**: 521-524.

Moayyedi, P.D. Forman, D. Braunholtz, R. Feltbower, W. Crocombe, M. Liptrott, and A. Axon on behalf of the Leeds HELP study group. 2000. The proportion of upper gastrointestinal symptoms in the community associated with *Helicobacter pylori*, lifestyle factors, and non-steroidal anti-inflammatory drugs. *Am. J. Gastroenterol.* **95**: 1449-1455.

Mobley, H.L.T., M.J. Cortesia, L.E. Rosenthal, and B.D. Jones. 1988. Characterization of urease from *Campylobacter pylori*. *J. Clin. Microbiol.* **26**: 831-836.

Mobley, H.L.T., R.E. Garner, and P.B. Bauerfeind. 1995. *Helicobacter pylori* nickel transport gene nixA: synthesis of catalytically active urease in *E. coli* independent of growth conditions. *Mol. Microbiol.* **16**: 97-109.

Molinari, M., C. Galli, M. de Bernard, N. Norais, J.M. Ruysschaert, R. Rappuoli, and C. Montecucco. 1998. The acid activation of *Helicobacter pylori* toxin VacA: structural and membrane binding studies. *Biochem. Biophys. Res. Commun.* **248**: 334-340.

Monstein H.J., A. Tiveljung, T.C.H. Kraft, K. Borch, and J. Jonasson. 2000. Profiling of bacterial flora in gastric biopsies from patients with *Helicobacter pylori*-associated gastritis and histologically normal control individuals by temperature gradient gel electrophoresis and 16S rDNA sequence analysis. *J. Med. Microbiol.* **49**: 817-822.

Montgomery, E.A., D.F. Martin, and D.A. Peura. 1988. Rapid diagnosis of *Hleicobacter pylori* by Gram's staining. *Am. J. Pathol.* **90**: 606-609.

Moore, R.A., B. Beckthold, S. Wong, A. Kureishi, and L.E. Bryan. 1995. Nucleotide sequence of the gyrA gene and characterization of the ciprofloxacin-resistant mutants of *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **39**: 107-111.

Moran, A.P. 1996. The role of lipopolysaccharide in *Helicobacter pylori* pathogenesis. *Ailment. Pharmacol. Ther.***10**: 39-50.

Moran, A.P. 2001. Molecular structure, biosynthesis and pathogenic roles of lipopolysaccharides. p. 81-95. In *Helicobacter pylori*: physiology and geneticseds. H.L.T. Mobley, G.L. Mendz, and S.L. Hazell. ASM Press, Washington DC.

Moran, A.P., and G.O. Aspinall. 1998. Unique structural and biological features of *Helicobacter pylori* lipopolysaccharides. *Prog. Clin. Biol. Res.* **397**: 37-49.

Morrel, A., B. Roth-Wicky, and F. Skvaril. 1983. Immunoglobulin subclass restriction of antibodies against hepatitis B surface antigen. *Infect. Immun.* **39**: 565-568.

Morshed, M.G., M. Karita, H. Konishi, K. Okita, and T. Nakazawa. 1994. Growth medium containing cyclodextrin and low concentration of horse serum for cultivation of *Helicobacter pylori*. *Microbiol. Immunol.* **38**: 897-900.

Morton, D. and K.D. Bardhan. 1995. Effect of transport medium and transportation time on culture of *Helicobacter pylori* from gastric biopsy specimens. *J. Clin. Pathol.* **48**: 91.

Mosman, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**: 55-59.

Mukhopadhyay, A.K., D. Kersulyte, J.Y. Jeong, S. Datta, Y. Ito, and A. Chaudhury. 2000. Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta, India. *Indian J. Bacteriol.* **182**: 3219-3227.

Mukhopadhyay, D.K., R.K. Tandon, S. Dasarathy, M. Mathur, and J.P. Wali. 1992. Study of *Helicobacter pylori* in North Indian subjects with non-ulcer dyspepsia. *Indian J. Gastroenterol.* **11**: 76-79.

Munoz, N. 1994. Is *Helicobacter pylori* a cause of gastric Cancer? An appraisal of the seroepidemiological evidence. *Cancer Epidemiol. Biomark. Prevent.* **3**: 445-451.

Muotiala, A., I.M. Helander, L. Pyhala, T.U. Kosunen, and A.P. Moran. 1992. Low biological activity of *Helicobacter pylori* lipopolysaccharide. *Infect. Immun.* **60**: 1714-1716.

Murakami, K., T. Fujioka, K. Shiota, A. Ito, K. Fujiyama, R. Kodama, Y. Kawasaki, T. Kubota, and M. Nasu. 1995. Influence of *Helicobacter pylori* infection and the effects of its eradication on gastric emptying in non-ulcer dyspepsia. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl. 1): S93-S97.

Murakita, H., M. Hirai, S. Ito, T. Azuma, T. Kato, and Y. Kohli. 1994. Vacuolating cytotoxin production by *Helicobacter pylori* isolates from peptic ulcer, atrophic gastritis, and gastric carcinoma patients. *Eur. J. Gastroenterol. Hepatol.* **6** (suppl 1): S29-S31.

Muszynski, J., D. Dzierzanowska, J. Sieminska, M. Bogdanska, E. Vogt, and A. Ehrmann. 1995. Is *Helicobacter pylori* infection a real risk factor for gastric carcinoma? *Scand. J. Gastroenterol.* **30**: 647-651.

Nakamura, T., S. Nakamura, M. Yonezumi, T. Suzuki, A. Matsuura, Y. Yatabe, T. Yokoi, K. Ohashi, and M. Seto. 2000. *Helicobacter pylori* and the (11;18) (q21;q21) translocation in gastric low-grade B-cell lymphoma of mucosa-associated lymphoid tissue type. *Jpn. J. Cancer Res.* **91**: 301-309.

Nakao, K., I. Imoto, N. Ikemura, T. Shibata, S. Takaji, Y. Taguchi, M. Misaki, K. Yamauchi, and N. Yamazaki. 1997. Relation of lactoferrin levels in gastric mucosa with *Helicobacter pylori* infection and with the degree of gastric inflammation. *Am. J. Gastroenterol.* **92**: 1005-1011.

Neil, G.A., and Ad Hoc Committee on FDA-related matters. 1997. Do ulcers burn out or burn on? Managing duodenal ulcer diathesis in the *Helicobacter pylori* era. *Am. J. Gastroenterol.* **92**: 387-393.

Ng, F.H., S.Y. Wong, W.F. Ng, and B.C.Y. Wong. 2000. Prolonged storage of frozen unbuffered rapid urease test. *J. Clin. Gastroenterol.* **31**: 142-143.

Nichols, L., M. Sughayer, P.C. DeGirolani, K. Baloch, D. Plesko, K. Eichelberger, and M. Santos. 1991. Evaluation of diagnostic methods for *Helicobacter pylori* gastritis. *Am. J. Pathol.* 95: 769-773.

Nielsen, H., S. Birkholz, L.P. Andersen, and A.P. Moran. 1994. Neutrophil activation by *Helicobacter pylori* lipopolysaccharides. *J. Infect. Dis.***170**: 135-139.

NIH Consensus Conference. 1994. *Helicobacter pylori* in peptic ulcer disease. *JAMA* **272**: 65-69.

Nishiya, D., T. Shimoyama, S. Fukuda, T. Yoshimura, M. Tanaka, and A. Munakata. 1999. Evaluation of the clinical relevance of the iceA1 gene in patients with *Helicobacter pylori* infection in Japan. *Scand. J. Gastroenterol.* **35**: 36-39.

Noach, L.A., T.M. Rolf, and G.N.J. Tytgat. 1994. Electronmicroscopic study of association between *Helicobacter pylori* and gastric and duodenal mucosa. *J. Clin. Pathol.* **47**: 699-704.

Nogueira, A.M.M.F., G.M. Ribeiro, M.A.G. Rodrigues, D.M.M. Queiroz, E.N. Mendez, G.A. Rocha, and A.J.A. Barbosa. 1993. Prevalence of *Helicobacter pylori* in Brazilian patients with gastric carcinoma. *Am. J. Clin. Pathol.* **100**: 236-239.

Nomura, A., and G.N. Stemmermann. 1993. *Helicobacter pylori* and gastric Cancer. *J. Gastroenterol. Hepatol.* **8**: 294-303.

Noorgaard, A., L.P. Andersen, and H. Nielsen. 1995. Neutrophil degranulation by *Helicobacter pylori* proteins. *Gut* **36**: 354-357.

Normansell, D.E. 1987. Human immunoglobulin subclasses. *Diag. Clin. Immunol.* **5**: 115-128.

Nydegger, U.E., and R.E. Butler. 1972. Serum lipoprotein levels in patients with Cancer. *Cancer Res.* **32**: 1756-1760.

O'Connor, H.J., C. Kanduru, A.S. Bhatta, J.M. Meehan, K.M. Feeley, and K. Cunnane. 1995. Effect of *Helicobacter pylori* eradication on peptic ulcer healing. *Postgrad. Med. J.* **71**: 90-93.

O'Toole, P.W., M.C. Lane, and S. Porwollik. 2000. *Helicobacter pylori* motility. *Microbe. Infect.* **2**: 1207-1214.

O'Toole, P.W., S.M. Logan, M. Kostrzynska, T. Wadstrom, and T.J. Trust. 1991. Isolation and Biochemical and Molecular analyses of a species-specific protein antigen from the gastric pathogen *Helicobacter pylori*. *J. Bacteriol.* **173**: 505-513.

Occhialini, A., M. Urdaci, F. Doucet-Populaire, C.M. Bebear, H. Lamouliatte, and F. Megraud. 1997. Macrolide resistance in *Helicobacter pylori*: rapid detection of point mutations and assays of macrolide binding to ribosomes. *Antimicrob. Agents. Chemother.* **41**: 2724-2728.

Ochs, H.D., and R.J. Wedgwood. 1987. IgG subclass deficiencies. *Ann. Rev. Med.* **38**: 325-340.

Ogihara, A., S. Kikuchi, A. Hasegawa, M. Kurosawa, K. Miki, E. Kaneko, and H. Mizukoshi. 2000. Relationship between *Helicobacter pylori* infection and smoking and drinking habits. *J. Gastroenterol. Hepatol.* **15**: 271-276.

Ohkura, R., H. Miwa, T. Murai, A. Nagahara, K. Ohta, K. Sato, T. Yamada, and N. Sato. 2000. Usefulness of a novel enzyme immunoassay for the detection of *Helicobacter pylori* in feces. *Scand. J. Gastroenterol.* **35**: 49-53.

Ohkusa, T., K. Fujiki, I. Takashmizu, J. Kumagai, T. Tanizawa, and Y. Eishi. 2000. Endoscopic and histological comparison of nonulcer dyspepsia with and without *Helicobacter pylori* infection evaluated by the modified Sydney system. *Am. J. Gastroenterol.* **95**: 2195-2199.

Olmos, J.A., H. Rios, R. Higa, and the Argentinean Hp epidemiologic study group. 2000. Prevalence of *Helicobacter pylori* infection in Argentina. Results of a nationwide epidemiologic study. *J. Clin. Gastroenterol.* **31**: 33-37.

Osaki, T., H. Yamaguchi, H. Taguchi, M. Fukada, H. Kawakami, H. Hirano, and S. Kamiya. 2002. Interleukin-8 induction and adhesion of the coccoid form of *Helicobacter pylori*. *J. Med. Microbiol.* **51**: 295-299.

Osaki, T.Y., H. Yamaguchi, H. Taguchi, S. Ogata, and S. Kamiya. 1995. Adherence of *Helicobacter pylori* to cultured human gastric carcinoma cells. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1) S89-S92.

Osato, M.S., K. Ayub, H.H. Le, R. Reddy, and D.Y. Graham. 1998. Housflies are unlikely reservoir or vector for *Helicobacter pylori*. *J. Clin. Microbiol.* **36**: 2786-2788.

Oshowo, A., D. Gillam, A. Botha, M. Tunio, J. Holton, P. Boulos, and M. Hobsley. 1998. *Helicobacter pylori*: the mouth, stomach and gut axis. *Ann. Periodontol.* **3**: 276-280.

Ota, H., and R.M. Genta. 1997. Morphological characterization of the gastric mucosa during infection with *H. pylori*, p.15-28. In P. Ernst, P. Michetti, and P.D. Smith (ed.), The immunology of *H. pylori*: from Pathogenesis to Prevention. Lippincott-Raven, Philadelphia.

Ottesen, E.A., F. Skvaril, S.P. Tripathy, R.W. Poindexter, and R. Hussain. 1985. Prominence of IgG_4 in the IgG antibody response to human filariasis. *J. Immunol.* **134**: 2707-2711.

Oxelius, V.A., A.B. Laurell, B. Lindquist, H. Golbiowska, U. Axelsson, J. Bjorkander and L.A. Hanson. 1981. IgG subclasses in selective IgA deficiency: Importance of IgG2-IgA deficiency. *N. Eng. J. Med.* **304**: 1476-1477.

Oxelius, V.A., A.I. Berkel, and L.A. Hanson. 1982. IgG_2 deficiency in ataxia-telangiectasia. *N. Eng. J. Med.* **306**: 515-517.

Palli, D., A. Decarli, F. Cipriani, F. Sitas, D. Forman, D. Amadori, C. Avellini, A. Giacosa, P. Manca, A. Russo, I.M. Samloff, J.F. Fraumeni, Jr., W.J. Blot, and E. Buiatti. 1993. *Helicobacter pylori* antibodies in areas of Italy at varying gastric Cancer risk. *Cancer Epidemiol. Biomark. Preven.* **2**: 37-40.

Palli, D., M. Galli, N.E. Caparaso, F. Cipriani, A. Decarli, C. Saieva, J.F. Fraumeni, Jr., and E. Buiatti. 1994. Family history and risk of stomach cancer in Italy. *Cancer Epidemiol. Biomarkers Prev.* **3**: 15-18.

Pan, Z.J., R.W.M. van der Hulst, M. Feller, S.D. Xiao, G.N.J. Tytgat, J. Dankert, and A. van der Ende. 1997. Equally high prevalence of infection with cagA positive *Helicobacter pylori* in Chinese patients with peptic ulcer disease and those with chronic gastritis-associated dyspepsia. *J. Clin. Microbiol.* **35**: 1344-1347.

Panella, C., E. Ierardi, L. Polimeno, T. Belzano, M. Ingrosso, A. Amaruso, A. traversa and A. Francavilla. 1996. Proliferative activity of gastric epithelium in progressive stages of *Helicobacter pylori* infection. *Dig. Dis. Sci.* **41**: 1132-1138.

Papadea, C., and I.J. Check. 1987. Immunoglobulin G subclasses. *CCN*: 10-13.

Pappo, J., S. Czinn, and J. Nedrud. 2001. Vaccines, p. 441-455. In *Helicobacter pylori*: Physiology and genetics.eds.H.L.T. Mobley, G.L. Mendz, and S.L. Hazell. ASM Press, Washington, DC.

Parsonnet, J. S. Hansen, L. Rodriguez, A.B. Gelb, R.A. Warnke, E. Jellum, N. Orentreich, J.H. Vogelman, and G.D. Friedman. 1994. *Helicobacter pylori* infection and gastric lymphoma. *N. Eng. J. Med.* **330**: 1267-1271.

Parsonnet, J., M. Replogle, S. Yang, and R. Hiatt. 1997. Seroprevalence of CagA-positive strains among *Helicobacter pylori*-infected healthy young adults. *J. Infect. Disease.* **175**: 1240-1242.

Parsonnet, J., G.D. Friedman, D.P. Vandersteen, Y. Chang, J.H. Vogelman, N. Orentreich, and R.K. Sibley. 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Eng. J. Med.* **325**: 1127-1131.

Pasceri, V., G. Cammarota, G. Patti, L. Cuoco, A. Gasbarrini, R.L. Grillo, G. Fedeli, G. Gasbarrini, and A. Maseri. 1998. Association of virulent *Helicobacter pylori* strains with ischaemic heart disease. *Circulation* **97**: 1675-1679.

Patel, P., M.A. Mendall, S. Khulusi, T.C. Northfield, and D.P. Strachan. 1994. *Helicobacter pylori* infection in childhood: risk factors and effect on growth. *Br. Med. J.* **309**: 1119-1123.

Pece, S., D. Fumarola, G. Giuliani, E. Jirillo, and A.P. Moran. 1995. Activity in the Limulus amoebocyte lysate assay and induction of tumour necrosis factor by diverse *Helicobacter pylori* lipopolysaccharide preparations. *J. Endotoxin Res.* **2**: 383- 390.

Pellicano, R., P.P. Parravicini, R. Bigi, N. Gandolfo, E. Aruta, V. Gai, N. Figura, P. Angelino, M. Rizzetto, and A. Ponzetto. 2002. Infection by *Helicobacter pylori* and acute myocardial infarction. Do cytotoxic strains make a difference? *Now. Microbiol.* **25**: 315-321.

Penfold, S.S., A.J. Lastovica, and B.G. Elisha. 1988. Demonstration of plasmids in *Campylobacter pylori*. *J. Infect. Dis.* **157**: 850.

Perez-Perez, G.I., A. Salomaa, T.U. Kousunen, B. Daverman, H. Rautelin, A. Aromaa, P. Knekt, and M.J. Blaser. 2002. Evidence that cagA$^+$ *Helicobacter pylori* strains are disappearing more rapidly than cagA- strains. *Gut* **50**: 295-298.

Perez-Perez, G.I., A.Z. Olivares, T.L. Cover, and M.J. Blaser. 1992. Characteristics of *Helicobacter pylori* variants selected for urease deficiency. *Infect. Immun.* **60**: 3658-3663.

Perez-Perez, G.I., B.M. Dworkin, J.E. Chodos, and M.J. Blaser. 1988. Campylobacter antibodies in humans. *Ann. Intern. Med.* **109**: 11-17.

Perez-Perez, G.I., V.L. Shepherd, J.D. Morrow, and M.J. Blaser 1995. Activation of human THP-1 cells and rat bone marrow-derived macrophages by *Helicobacter pylori* lipopolysaccharide. *Infect. Immun.* **63**: 1183-1187.

Peterson, R.A., T. Hoepf, and K.A. Eaton. 2003. Adoptive transfer of splenocytes in SCID mice implicates CD4$^+$ T-cells in apoptosis and epithelial proliferation associated with *Helicobacter pylori*-induced gastritis. *Comp. Med.* **53**: 498-509.

Peura, D.A. 1997. Ulcerogenesis: Integrating the role of *Helicobacter pylori* and acid secretion in duodenal ulcer. *Am. J. Gastroenterol.* **92**: 8S-14S.

Piccolomini, R., G.D. Bonaventura, D. Festi, G. Catamo, F. Laterza, and M. Neri. 1997. Optimal combination of media for primary isolation of *Helicobacter pylori* from gastric biopsy specimens. *J. Clin. Microbiol.* **35**: 1541-1544.

Pieniazek, P., E. Karezewska, A. Duda, W. Tracz, M. Pasowicz, and S.J. Konturek. 1999. Association of *Helicobacter pylori* infection with coronary heart disease. *Physiol. Pharmacol.* **50**: 743-751.

Pietroiusti, A., M. Diomedi, L.M. Cupini, I. Luzzi, M.J. Gomez-Miguel, A. Bergamaschi, A. Magrini, T. Carrabs, M. Vellini, and A. Galante. 2002. Cytotoxin-associated gene-A-positive *Helicobacter pylori* strains are associated with atheroscerotic stroke. *Circulation* **106**: 580-584.

Prabhu, S.R., S. Ranganathan, and D.N. Amarapurkar. 1994. *Helicobacter pylori* in normal gastric mucosa. *J. Assoc. Physicians India* **42**: 863-864.

Pronovost, A.D., S.L. Rose, J.W. Pawlak, H. Robin, and R. Schneider. 1994. Evaluation of new immunodiagnostic assay for *Helicobacter pylori* antibody detection: Correlation with hisopathological and microbiological results. *J. Clin. Microbiol.* **32**: 46-50.

Proujansky, R., S.E. Shaffer, N.E. Vinton, and S.J. Bachrach. 1994. Symptomatic *Helicobacter pylori* infection in young patients with severe neurologic impairment. *J. Paeditr.* **125**: 750-752.

Queiroz, D.M.M., E.N. Mendes, and G.A. Rocha. 1987. Indicator medium for isolation of *Campylobacter pylori*. *Eur. J. Clin. Microbiol.* **25**: 2378-2379.

Queiroz, D.M.M., E.N. Mendez, A.S.T. Carvalho, G.A. Rocha, A.M.R. Oliveira, T.F. Soares, A. Santos, M.M.D.A. Cabral, and A.M.M.F. Nogueira. 2000. Factors associated with *Helicobacter pylori* infection by a cagA-positive strain in children. *J. Infect. Dis.* **181**: 626-630.

Radcliff, F.J., S.L. Hazell, T. Kolesnikow, C. Doidge, and A. Lee. 1997. Catalase, a novel antigen for *Helicobacter pylori* vaccination. *Infect. Immun.* **65**: 4668-4674.

Ramarao, N., and T.F. Meyer. 2001. *Helicobacter pylori* resists phagocytosis by macrophages: Quantitative assessment by confocal microscopy and fluorescence-activated cell sorting. *Infect. Immun.* **69**: 2604-2611.

Ramarao, N., S.D. Gray-Owen, and T.F. Meyer. 2000. *Helicobacter pylori* inhibits phagocytosis by professional phagocytes by involving type IV secretion components. *Mol. Microbiol.* **37**: 1389-1404.

Rankin, S., M.A. Parthasarathy, and D. Steinberg. 1991. Evidence for a dominant role of lipooxygenase(s) in the oxidation of LDL by mouse peritoneal macrophages. J. *Lipid Res.* 32: 449-456.

Rasko, D.A., J.M. T.J.M. Wilson, D. Zopf, and D.E. Taylor. 2000. Lewis antigen expression and stability in *Helicobacter pylori* isolated from serial gastric biopsies. *J. Infect. Dis.* **181**: 1089-1095.

Rathbone, B.J., J.I. Wyatt, B.W. Worsley, S.E. Shires, L.K. Trejdosiewicz, R.V. Heatley, and M.S. Lowosky. 1986. Systemic and local antibody response to gastric *Campylobacter pyloridis* in non-ulcer dyspepsia. *Gut* **27**: 642.

Rautelin, H., and T.U. Kosunen. 1991. *Helicobacter pylori* and associated gastroduodenal diseases. *APMIS* **99**: 677-695.

Rautelin, H., B. Blomberg, H. Fredlund, G. Jemerot, and D. Danielsson. 1993. Incidence of *Helicobacter pylori* strains activating neutrophils in patients with peptic ulcer disease. *Gut* **34**: 599-604.

Raymond, J., M. Bergeret, P.H. Benhamou, K. Mensah, and C. Dupont. 1994. A 2-year study of *Helicobacter pylori* in children. *J. Clin. Microbiol.* **32**: 461-463.

Reinhard, J., C. Basset, J. Holton, M. Binks, P. Youinou, and D. Vaira. 2000. Image analysis method to assess adhesion of *Helicobacter pylori* to gastric epithelium using confocal laser scanning microscopy. *J. Microbiol. Method* **39**: 179-187.

Reisen, W.F., F. Skvaril, and D.G. Braun. 1976. Natural infection of man with groupA Streptococci: levels, restriction in class, subclass, and type, and clonal appearance of polysaccharide group specific antibodies. *Scand. J. Immunol.* **5**: 383-390.

Rifai, N., P.S. Bachorik, and J. Albers. 1999. Lipids, lipoproteins, and apolipoproteins. p. 809-861. In Teitz textbook of clinical chemistry. IIIrd edition.eds.C.A. Burtis, and E.R. Ashwood. W.B. Saunders and Company.

Roggero, E., E. Zucca, G. Pinotti, A. Pascerella, C. Capella, A. Savio, E. Pedrinis, A. Peterlini, A. Venco, and F. Cavelli. 1995. Eradication of *Helicobacter pylori* infection in primary low-grade gastric lymphoma of mucosa associated lymphoid tissue. *Ann. Intern. Med.* **122**: 767-769.

Rokkas, T., G. Papatheodorou, A. Karameris, A. Mavrogeorgis, N. Kalogeropoulos, and N. Giannikos. 1995. *Helicobacter pylori* infection and gastric juice vitamin C levels: Impact of eradication. *Dig. Dis. Sci.* **40**: 615-621.

Romagnani, S., P. Parronchi, M. D'Elios, P.Romagnani, F. Annunziato, M. Menghetti, E. Maggi, and G. DelPrete. 1996. Immune mechanisms in the pathogenesis of inflammatory gastrointestinal disorders. *Itl. J. Gastroenterol.* **28** (suppl. 2): 11-17.

Rose, G, and M.S. Shipley. 1980. Plasma lipids and mortality: a source of error. *Lancet* **1**: 523-526.

Rossi, G., D. Fortuna, L. Pancotto, G. Renzoni, E. Taccini, P. Ghiara, R. Rappuoli, and G.D. Guidice. 2000. Immunohistochemical study of lymphocyte populations infiltrating the gastric mucosa of Beagle dogs experimentally infected with *Helicobacter pylori*. *Infect. Immun.* **68**: 4769-4772.

Rubenstein, G., K. Dunkin, and A.J. Howard. 1994. The susceptibility of *Helicobacter pylori* to 12 antimicrobial agents, omeprazole and bismuth salts. *J. Antimicrobial Chemother.* **34**: 409-413.

Rudi, J., M. Muller, A. von Herbay, I. Zuna, R. Raedsch, W. Stremmel, and U. Rath. 1995. Lack of association of *Helicobacter pylori* seroprevalence and gastric *Cancer* in the population with low gastric *Cancer* incidence. *Scand. J. Gastroenterol.* **30**: 958-963.

Saitoh, T., H. Natomi, W. Zhao, K. Okuzumi, K. Sugano, M. Iwamori and Y. Nagai. 1991. Identification of glycolipid receptors for *Helicobacter pylori* by TLC-immunostaining. *FEBS Letters* **282**: 385-387.

Sakaki, N. 1995. The influence of *Helicobacter pylori* infection on the progression of gastric mucosal atrophy and occurrence of gastric Cancer. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl. 1): S59-S62.

Sakaki, N., k. Momma, N. Egawa, Y. Yamada, T. Kan, and J. Ishiwata. 1995. The influence of *Helicobacter pylori* infection on the progression of gastric mucosal atrophy and occurrence of gastric Cancer. *Eur. J. Gastroenterol. Hepatol.* **7** (Suppl 1): S59-S62.

Sakamoto, J., and K.O. Llyoyd. 1989. Expression of Lewis^a, Lewis^r, Lewis^b, sialyl-Lewis^a, and sialyl-Lewis^x blood group antigens in human gastric carcinoma and in normal gastric tissue. *Cancer Res.* **49**: 745-752.

Salim, A.S. 1994. Oxygen-derived free-radical scavengers prolong survivial in gastric Cancer. *Chemother.* **35**: 179-185.

Samesto, A., S. Ranta, P. Vaananen, P., and O. Makela. 1985. Proportion of immunoglobulin classes and subclasses in rubella antibodies. *Scand. J. Immunol.* **21**: 275-282.

Sartor, R.B. 2002. Mucosal immunology and mechanism of gastrointestinal inflammation. p. 21-51. In Sleisenger and Fordtran's gastrointestinal and liver diseases: Pathophysiology, diagnosis and management.eds.M. Feldman, L.S. Friedman, and M.H. Sleisenger Vol. I. Saunders edn.

Satoh, K., K. Kimura, and P. Sipponen. 1995. *Helicobacter pylori* infection and chronological extension of atrophic gastritis. *Eur. J. Gastroenterol. Hepatol.* **7** (suppl 1) S11-S15.

Schur, P.H., H. Borel, E.W. Gelfand, C.A. Alper, and F.S. Rosen. 1970. Selective gamma G globulin deficiencies in patients with recurrent pyogenic infections. *N. Eng. J. Med.* **283**: 631-634.

Scotiniotis, I.A., T. Rokkas, E.E. Furth, B. Rigas, and S.J. Shiff. 2000. Altered gastric epithelial cell kinetics in *Helicobacter pylori*-associated intestinal metaplasia: Implications for gastric Carcinogenesis. *Int. J. Cancer.* **85**: 192-200.

Scott, D.R., E.A. Marcus, D.L. Weeks, A. Lee, K. Melchers, and G. Sachs. 2000. Expression of the *Helicobacter pylori* urel gene is required for acidic pH activation of cytoplasmic urease. *Infect. Immun.* **68**: 470-477.

Scott, M.G., and M.H. Nahm. 1984. Mitogen-induced human IgG subclass expression. *J. Immunol.* **135**: 2454-2460.

Scott, M.G., M.H. Nahm, K. Macke, G.S. Nash, M.J. Bertovich, and R.P. Mac Dermott. 1986. Spontaneous secretion of IgG subclasses by intestinal mononuclear cells: differences between ulcerative colitis, Crohn's disease, and controls. *Clin. Exp. Immunol.* **66**: 209-215.

Sell, S. 1987. Immunology, Immunopathology and immunity. 4th Edn. p. 261-306. Elsevier New York.

Shackelford, P.G., S.H. Polmar, J.L. Mayus, W.L. Johnson, J.M. Corry, and M.H. Nahm. 1986. Spectrum of IgG2 subclass deficiency in children with recurrent infections: Prospective study. *J. Paediatr.* **108**: 647-653.

Shakib, F., D.R. Stanworth, C.A. Smally, and G.A. Brown. 1976. Elevated serum IgG_4 levels in cystic fibrosis. Clin. *Allergy* **6**: 237-239.

Shakib, F., D.R. Stanworth, R. Drew, and D. Catty. 1975. A quantitative study of the distribution of IgG subclasses in a group of normal human sera. *J. Immunol. Methods* **8**: 17-28.

Shakib, F., P. McLaughlan, D.R. Stanworth, E. Smith, and E. Fairburn. 1977. Elevated serum IgE and IgG_4 in patients with atopic dermatitis. *Br. J. Dermatol.* **97**: 59-63.

Sherburne, R, and D.E. Taylor. 1995. *Helicobacter pylori* expresses a complex surface carbohydrate, Lewis ^{x}. *Infect. Immun.* **63**: 4564-4568.

Shimoyama, T., S. Fukuda, M. Tanaka, N. Sugawara, F. Kurimoto, and A. Munakata. 2000. Serum anti-Lewis x antibody is not elevated in patients with gastric Cancer infected with *Helicobacter pylori*. *J. Clin. Gastroenterol.* **31**: 48-50.

Shimoyama, T., S. Fukuda, M. Tanaka, S. Nakaji, and A. Munakata. 2000. Evaluation of the applicability of the gastric carcinoma risk index for intestinal type *Cancer* in Japanese patients infected with *Helicobacter pylori*. *Virchows Arch.* **436**: 585-587.

Shimuzu, N., K. Inada, H. Nakanishi, T. Tsukamoto, Y. Ikehara, M. Kaminishi, and T. Tatematsu. 1999. *Helicobacter pylori* infection enhances glandular stomach *Carcinogenesis* in Mongolian gerbil treated with chemical carcinogens. *Carcinogenesis* **20**: 669-676.

Shirai, M., J. Kakada, K. Shibata, M.G. Morshed, T. Matsushita, and T. Nakzawa. 2000. Accumulation of polyphosphate granules in *Helicobacter pylori* cells under anaerobic conditions. *J. Med. Microbiol.* **49**: 513-519.

Shirai, Y., Y. Wakatsuki, T. Kusumoto, M. Nakata, M. Yoshida, T. Usui, T. Iizuka, and T. Kita. 2000. Induction and maintenance of immune effector cells in the gastric tissue of mice orally immunized to *Helicobacter pylori* requires salivary glands. *Gastroenterol.* **118**: 749-759.

Siber, G.R., P.H. Schur, A.C. Aisenberg, S.A. Weitzman, and G. Schiffman. 1980. Correlation between serum IgG2 concentrations and the antibody response to bacterial polysaccharide antigens. *N. Eng. J. Med.* **303**: 178-182.

Sipponen, P. 1992. *Helicobacter pylori* infection-A common worldwide environmental risk factor for gastric *Cancer*? *Endoscopy* **24**: 424-427.

Sipponen, P. 1992. Natural history of gastritis and its relationship to peptic ulcer disease. *Digestion* **51** (suppl 1): 70-75.

Sipponen, P. 1994. Gastric *Cancer*- A long-term cosequence of *Helicobacter pylori* infection. *Scand. J. Gastroenterol.* **29** (Suppl201) 24-27.

Sipponen, P. 1995. *Helicobacter pylori*: a cohort phenomenon. *Am. J. Sur. Pathol.* **19** (suppl 1): S30-S36.

Sipponen, P., and K. Kimura. 1994. Intestinal metaplasia, atrophic gastritis, and stomach *Cancer*: Trends over time. *Eur. J. Gastroenterol. Hepatol.* **6** (suppl 1): S79-S83.

Sipponen, P. T. Helske, P. Jarvinen, H. Hyvarinen, K. Seppala, and M. Siurala. 1994. Fall in the prevalence of chronic gastritis over 15 years: analysis of out patients series in Finland from 1977, 1985 and 1992. *Gut* **35**: 1167-1171.

Sipponen, P., J. Valle, K. Varis, M. Kekki, T. Ihamaki, and M. Siurala. 1990. Fasting levels of serum gastrin in different functional and morphologic states of the antrofundal mucosa. An analysis of 860 subjects. *Scand. J. Gastroenterol.* **25**: 513-519.

Sipponen, P., M. Riihela, H. Hyvarinen, and K. Seppala. 1994. Chronic nonatrophic ('superficial') gastritis increases the risk of gastric carcinoma: a case-control study. *Scand. J. Gastroenterol.* **29**: 336-340.

Sipponen, P., T.U. Kousunen, J. Valle, M. Riihela, and K. Seppala. 1992. *Helicobacter pylori* and chronic gastritis in gastric *Cancer*. *J. Clin. Pathol.* **45**:319-323.

Sjunnesson, H., E. Sturegard, S. Hynes, R. Willen, R. Feinstein, and T. Wadstrom. 2003. Five month persistence of *Helicobacter pylori* infection in guinea pigs. *APMIS* **111**: 634-642.

Slomiany, B.L., J. Piotrowsky, and A. Slomiany. 1998. Induction of caspase 3 and nitric oxide synthase 2 during mucosal inflammatory reaction to *Helicobacter pylori* lipopolysaccharide. *Biochem. Mol. Biol. Int.* **46**: 1063-1070.

Slomiany, B.L., Y.H. Liau, R.A. Lopez, J. Piotrowski, A. Czajkowski, and A. Slomiany. 1992. Effect of *Helicobacter pylori* lipopolysaccharide on the synthesis of sulphated gastric mucin. *Biochem. International* **27**: 687-697.

Smith, A.W., B. Chahal, and G.L. French. 1994. The human gastric pathogen *Helicobacter pylori* has a gene encoding an enzyme first classified as a mucinase in *Vibrio cholerae*. *Mol. Microbiol.* **13**: 153-160.

Smith, G.V., A.P. Moran, M.B. Ellitot, and M.J. Farthing. 2003. Induction of cyclooxygenase 2 by *Escherichia coli* but not Helicobacter pylori lipopolysaccharide in gastric epithelial cells in vitro. *Helicobacter* **8**: 513-520.

Smoot, D.T., H.L.T. Mobley, G.R. Chippendale, J.F. Lewison, and J.H. Resau. 1990. *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. *Infect. Immun.* **58**: 1992-1994.

Smoot, D.T., Z. Wynn, T.B. Elliott, C.R. Allen, G. Mekasha, T. Naab, and H. Ashktorab. 1999. Effect of *Helicobacter pylori* on proliferation of gastric epithelial cells in vitro. *Am. J. Gastroenterol.* **94**: 1508-1511.

Sobala, G.M., C.J. Schorah, S. Shires, D.A.F. Lynch, B. Gallacher, M.F. Dixon, and A.T.R. Axon. 1993. Effect of eradication of *Helicobacter pylori* on gastric juice ascorbic acid concentrations. *Gut* **34**: 1038-1041.

Sobala, G.M., J. Crabtree, M.F. Dixon, C.J. Schorah, J.D. Taylor, B.J. Rathbone, R.V. Heatley, and A.T.R. Axon. 1991. Acute *Helicobacter pylori* infection: clinical features, local and systemic immune response, gastric mucosal histology, and gastric juice ascorbic acid concentrations. *Gut* **32**: 1415-1418.

Solca, N.M., M.V. Bernasconi, and J.C. Piffaretti. 2000. Mechanism of metronidazole resistance in *Helicobacter pylori*: Comparison of the rdxA gene sequences in 30 strains. *Antimicrob. Agents Chemother.* **44**: 2207-2210.

Song, Q., T. Lange, A. Spahr, G. Adler, and G. Bode. 2000. Characteristic distribution pattern of *Helicobacter pylori* in dental plaque and saliva detected with nested PCR. *J. Med. Microbiol.* **49**: 349-353.

Spigelhalder, C., B. Gerstenecker, A. Kersten, E. Schiltz, and M. Kist. 1993. Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. *Infect. Immun.* **61**: 5315-5325.

Stacey, A.R., P.R. Hawtin, and D.G. Newell. 1990. Antigenicity of fractions of *Helicobacter pylori* prepared by fast protein liquid chromatography and urease captured by monoclonal antibodies. *Eur. J. Clin. Microbiol. Infect. Dis.* **9**: 732-737.

Stanley, P.J., G. Corbo, and P.J. Cole. 1984. Serum IgG subclasses in chronic and recurrent respiratory infections. *Clin. Exp. Ommunol.* **58**: 703-708.

Stanworth.D.R. 1983. Immunological aspects of human IgG4. *Clin. Rev. Allerg.* **1**: 183-195.

Steel, K.J. 1961. The oxidase reaction as a taxonomic tool. J. *Gen. Microbiol.* **25**: 297-306.

Stein, M., R. Rappuoli, and A. Covacci. 2001. The cag pathogenicity island. p. 345-353. In *Helicobacter pylori*: Physiology and genetics.eds.H.L.T. Mobley, G.L. Mendz, and S.L. Hazell. ASM Press, Washington, DC.

Steinberg, D. and J.L. Witztum. 1990. Lipoproteins and atherogenesis: current concepts. *JAMA* **264**: 3047-3052.

Steinberg, D., S. Parthasarathy, T.E. Carew, J.D. Khoo, and J.L. Witztum. 1989. Beyond cholesterol: modifications of low density lipoprotein that increase its atherogenicity. *N. Eng. J. Med.* **320**: 915-924.

Steininger, H., G. Faller, E. Dewald, T. Brabletz, A. Jung, and T. Kirchner. 1998. Apoptosis in the chronic gastritis and its correlation with antigastric autoantibodies. *Virchows Arch.* **433**: 13-18.

Steinman, R.M., I.S. Mellman, W.A. Mueller, and Z.A. Cohn. 1983. Endocytosis and recycling of plasma membrane. *J. Cell Biol.* **96**: 1-27.

Stevens, R., D. Dicher, B. Keld, and D. Heiner. 1983. IgG_1 is the predominant subclass of *in vivo* and *in vitro* produced anti-tetanus toxoid antibodies and also serves as the membrane IgG molecule for delivering inhibitory signals to anti-tetanus toxoid antibody producing B cells. *J. Clin. Immunol.* **3**: 65-70.

Stolte, M., E. Bayerdorffer, A. Morgner, B. Alpen, T. Wundisch, C. Thiede, and A. Neubauer. 2002. *Helicobacter* and gastric MALT lymphoma. *Gut* **50** (suppl III): iii19-iii24.

Su, B., P.J. Ceponis, and P.M.Sherman. 2003. Cytoskeletal rearrangements in gastric epithelial cells in response to *Helicobacter pylori* infection. *J. Med. Microbiol.* **52**: 861-867.

Suerbaum, G.G., S. Forthoff, B. Leying, and W. Opferkuch. 1993. Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*. *J. Med. Microbiol.* **38**: 371-377.

Sugiyama, A., F. Maruta, T. Ikeno, K. Ishida, S. Kawasaki, T. Katsuyama, N. Shimizu, and M. Tatematsu. 1998. *Helicobacter pylori* infection enhances N-methyl-N-nitrosourea-induced stomach *Carcinogenesis* in Mongolian gerbil. *Cancer Res.* **58**: 2067-2069.

Sullivan, P.B., and J.E. Thomas. 1991. *Helicobacter pylori* in children. *Postgrad. Med. J.* **67**: 330-333.

Sullivan, P.B., J.E. Thomas, D.G.D. Wight, G. Neale, E.J. Eastham, T. Corrah, N.L. Evans, and B.M. Greenwood. 1990. *Helicobacter pylori* in Gambian children with chronic diarrhoea and malnutrition. *Arch. Dis. Childhood.* **65**: 189-191.

Sumii, M., K. Sumii, A. Tari, H. Kawaguchi, G. Yamamoto, Y. Takehara, Y. Fukino, T. Kamiyasu, M. Hamada, and T. Tsuda. 1994. Expression of antral gastrin and somatostatin mRNA in *Helicobacter pylori* infected subjects. *Am. J. Gastroenterol.* **89**: 1515-1519.

Sundqvist, V.A., A. Linde, and B. Wahren. 1984. Virus-specific immunoglobulin G subclasses in herpes simplex and varicella-zoster virus infections. *J. Clin. Microbiol.* **20**: 94-98.

Suzuki, H., and H. Ishii. 2000. Role of apoptosis in *Helicobacter pylori*-associated gastric mucosal injury. *J. Gastroenterol. Hepatol.* **15** (suppl): D46-D54.

Suzuki, M., S. Miura, M. Suematsu, D. Fukumura, I. Kurose, H. Suzuki, A. Kai, Y. Kudoh, M. Ohashi, and M. Tsuchiya. 1992. *Helicobacter pylori* associated ammonia production enhances neutrophil dependent mucosal cell injury. *Am. J. Physiol.* **263**: G719-G725.

Takahashi, S.I., H. Igarashi, N. Ishiyama, K. Nakamura, M. Masubuchi, M. Ozaki, S. Saito, T. Aoyagi, T. Itoh and I. Hrata. 1993. Is *Helicobacter pylori* a causal agent in gastric carcinoma? *Zbl. Bakt.* **280**: 144-149.

Tanahashi, T., M. Kita, T. Kodama, Y. Yamaoka, N. Sawai, T. Ohno, S. Mitsufuji, Y.-P. Wei, K. Kashima, and J. Imanishi. 2000. Cytokine expression and production by purified *Helicobacter pylori* urease in human gastric epithelial cells. *Infect. Immun.* **68**: 664-671.

Tanigawa, T., T. Kawamori, M. Iimuro, T. Ohta, K. Higuchi, T. Arakawa, T. Sugimura, and K. Wakabayashi. 2000. Marked enhancement by fish meal of *Helicobacter pylori*-induced gastritis in Mongolian gerbils. *Jpn. Cancer Res.* **91**: 769-773.

Tankovic, J., D. Lamarque, J.C. Delheir, C.J. Soussy, A. Labigne, and P.J. Jenks. 2000. Frequent association between alteration of the rdxA gene and metronidazole resistance in French and North African isolates of *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **44**: 608-613.

Tatsuguchi, A., C. Sakamoto, K. Wada, T. Akamatsu, T. Tsukui, K. Miyake, S. Futagami, T. Kishida, Y. Fukuda, N. Yamanama, and M. Kobayashi. 2000. Localization of cyclooxygense 1 and cyclooxygenase 2 in *Helicobacter pylori* related gastritis and gastric ulcer tissues in humans. *Gut* **46**: 782-789.

Taylor, D.E. 1988. Campylobacter pylori- Isolation, characterization, and association with chronic gastritis and ulcers. *Culture*. **9**: 10-13.

Taylor, D.N., and M.J. Blaser. 1991. The epidemiology of *Helicobacter pylori* infection. *Epidemiologic Reviews* **13**: 42-59.

Taylor, W.I., and D. Achanzar. 1972. Catalase test as an aid to the identification of Enterobacteriaceae. *Apllied Micorbiol.* **24**: 58.

Telford, J.L., A. Covacci, P. Ghiara, C. Montecucco, and R. Rappuoli. 1994. Unravelling the pathogenic role of *Helicobacter pylori* in peptic ulcer: potential new therapies and vaccines. *TIBTECH* **12**: 420-426.

Terry, W.D., and J.L. Fashey. 1964. Subclasses of human gamma globulin based on difference in the heavy peptide chains. *Science* **146**: 400-401.

Testerman, T.L., D.J. McGee, and H.L.T. Bobley. 2001. *Helicobacter pylori* growth and urease detection in the chemically defined medium Ham's F-12 nutrient mixture. *J. Clin. Microbiol.* **39**: 3842-3850.

Testoni, P.A., and F. Bagnolo. 2000. In dyspeptic patients without gastric phase III of the migrating motor complex, *Helicobacter pylori* eradication produces no short-term changes in interdigestive motility pattern. *Scand. J. Gastroenterol.* **35**: 808-813.

Thomas, J.E., G.R. Gibson, M.K. Darboe, A. Dale, and L.T. Weaver. 1992. Isolation of *Helicobacter pylori* from human faeces. *Lancet* **340**: 1194-1195.

Tjia, T.N., W.E.S. Harper, C.S. Goodwin, and W.B. Grub. 1987. Plasmids in *Campylobacter pyloridis*. *Microbios Lett.* **36**: 7-11.

Tokunaga, Y., H. Shirahase, E. Yamamoto, R. Inao, S. Hamaguchi, K. Kanaji, A. Kitaoka, T. Yagi, A. Tokuka, and K. Ohsumi. 2000. Modified urease test for *Helicobacter pylori* detection in relation to an immunohistochemical stain. *J. Gastroenterol. Hepatol.* **15**: 617-621.

Tokunaga, Y., H. Shirahase, E. Yamamoto, Y. Gouda, K. Kanaji, and K. Ohsumi. 1998. Semiquantitative evaluation of diagnosis of *Helicobacter pylori* infection in relation to histological changes. *Am. J. Gastroenterol.* **93**: 26-29.

Tomb, J.F., O. White, A.R. Kerlavage, R.A. Clayton, G.G. Sutton, R.D. Fleischman, K.A. Ketchum, H.P. Klenk, S. Gill, B.A. Doughetry, K. Nelson, J. Quackenbush, L. Zhou, E.F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H.G. Khalak, A.Glodek, K. McKenney, L.M. Fitzegerald, N. Lee, M.D. Adams, E.K. Hickey, D.E. Berg, J.D. Gocayne, T.R. Utterback, J.D. Peterson, J.M. Kelley, M.D. Cotton, J.M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W.S. Hayes, M. Borodovsky, P.D. Karp, H.O. Smith, C.M. Fraser, and J.C. Venter. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**: 539-547.

Trebesius, K., K. Panthel, S. Strobel, K. Vogt, G. faller, T. Kirchnen, M. Kist, J. Heesemann, and R. Haas. 2000. Rapid and specific detection of *Helicobacter pylori* macrolide resistance in gastric tissue by fluorescence in situ hybridisation. *Gut* **46**: 608-614.

Tsuda, M., M. Karita, M.G. Morshed, K. Okita, and T. Nakasaki. 1994. A urease negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. *Infect. Immun.* **62**: 3586-3589.

Tsugane, S., M. Kabuto, H. Imai, F. Gey, Y. Tei, T. Hanaoka, K. Sugano, and S. Watanabe. 1993. *Helicobacter pylori*, dietary factors, and atrophic gastritis in five Japanese populations with different gastric *Cancer* mortality. *Cancer Causes Contr.* **4**: 297-305.

Tursi, A., G. Cammarota, A. Papa, L. Cuoco, G. Fedeli, and G. Gasbarrini. 1997. Long-term follow-up of disappearance of gastric mucosa-associated lymphoid tissue after anti-*Helicobacter pylori* therapy. *Am. J. Gastroenterol.* **92**: 1849-1853.

Tytgat, G.N.J. 2000. Ulcers and gastritis. *Endoscopy* **32**: 108-117.

Uemura, N., S. Okamoto, S. Yamamoto, N. Matsumura, S. Yamaguchi, M. Yamakido, K. Taniyama, N. Sasaki, and R.J. Schlemper. 2001. *Helicobacter pylori* infection and the development of gastric *Cancer*. *N. Eng. J. Med.* **345**: 784-789.

Urbanek, R., D.M. Kemeny, and D. Richards. 1986. Subclass of IgG anti-bee venom antibody produced during bee venom immunotherapy and its relation to the long-term protection from bee stings and following termination of venom immunotherapy. *Clin. Allergy* **16**: 317-322.

Vaezi, M.F., G.W. Falk, R.M. Peek, J.J. Vicari, J.R. Goldblum, G.I. Perez-Perez, T.W. Rice, M.J. Blaser, and J.E. Richter. 2000. CagA-positive strains of *Helicobacter pylori* may protect against Barrett's esophagus. *Am. J. Gastroenterol.* **95**: 2206-2211.

Vaira, D., P. Malfertheiner, F. Megraud, A.T. Axon, M. Deltenre, A.M. Hirschl, G. Gasbarrini, C. O'Morain, J.M. Garcia, M. Quina, and G.N. Tytgat. 1999. Diagnosis of *Helicobacter pylori* infection with a new non-invasive antigen-based assay. HpSA European study group. *Lancet* **354**: 30-33.

Vakil, N., and M.F. Go. 2000. Treatment of Helicobacter *pylori* infection. *Curr. Opin. Gastroenterol.* **16**: 32-39.

Valle, J., M. Kekki, P. Sipponen, P. Sipponen, T. Ihamaki, and M. Siurala. 1996. Long-term course and consequences of *Helicobacter pylori* gastritis. *Scand. J. gastroenterol.* **31**: 546-550.

van den Brink, G.R., K.M.A.J. Tytgat, R.W.M. van der Hulst, C.M. van der Loos, A.W.C. Einerhand, H.A. Buller, and J. Dekker. 2000. *H. pylori* colocalises with MU5AC in the human stomach. *Gut* **46**: 601-607.

van der Ende, A. L.J. van Doorn, S. Rooijakkers, M. Feller, G.N.J. Tytgat, and J. Dankert. 2001. Clarithromycin-susceptible and–resistant *Helicobacter pylori* isolates with identical randomly amplified polymorphic DNA-PCR genotypes cultured from single gastric biopsy specimen prior to antibiotic therapy. *J. Clin. Microbiol.* **39**: 2648-2651.

van Doorn, L.J., Y. Henskens, N. Nouhan, A. Verschuuren, R. Vreede, P. Herbink, G. Ponjee, K. van Krimpen, R. Blankenburg, J. Scherpenisse, and W. Quint. 2000. The efficacy of laboratory diagnosis of *Helicobacter pylori* infections in gastric biopsy specimens is related to bacterial density and vacA, cagA, and iceA genotypes. *J.Clin. Microbiol.* **38**: 13-17.

Versalovic, J. D. Shortridge, K. Kliber, V. griffy, J. Beyer, R.K. Flamm, S.K. tanaka, D.Y. Graham, and M.F. Go. 1996. Mutaions in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **40**: 477-480.

Vitols, S., B. Angelin, S. Ericsson, G. Garhton, G. Juliusson, M. Masquelier, C. Paul, C. Peterson, M. Rudling, K. Sodenberg-Reid. 1990. Uptake of low-density lipoprotein by human leukemic cells in vivo: relation to plasma lipoprotein levels and possible relevance for selective chemotherapy. *Proc. Natl. Acad. Sci. USA.* **87**: 2598-2602.

Vitols, S., G. Gahrton, M. Bjorkholn, and C. Petersen. 1985. Hypocholesterolemia in malignancy due to elevated low-density-lipoprotein-receptor activity in tumour cells: evidence from studies in patients with leukaemia. *Lancet* **2**: 1150-1154.

Vollmers, H.P., J. Dammrich, H. Ribbert, S. Grassel, S. Debus, J. Heesemann, and H.K.M. Hermelink. 1994. Human monoclonal antibodies from stomach carcinoma patients react with *Helicobacter pylori* and stimulate stomach *Cancer* cells *in vitro*. *Cancer* **74**: 1525-1532.

von Wulffen, H. J. Heesemann, G.H. Butzow, T. Loning, and R. Laufs. 1986. Detection of *Campylobacter pyloridis* in patients with antrum gastritis and peptic ulcer by culture, complement fixation test and immunoblot. *J. Clin. Microbiol.* **24**: 716-720.

von Wulffen, H., H.J. Grote, S. Gatermann, T. Loning, B. Berger, and C. Buhl. 1988. Immunoblot analysis of immune response to *Campylobacter pylori* and its clinical associations. *J. Clin. Pathol.* **41**: 653-659.

von Wullfen, H., and H.J. Grote. 1988. Enzyme linked immunosorbent assay for detection of immunoglobulin A and G antibodies to *Campylobacter pylori*. *Eur. J. Clin. Microbiol. Infect. Dis.* **7**: 559-565.

Vorobjova, T., G. Faller, H.I. Maaroos, P. Sipponen, K. Villako, R. Uibo, and T. Kirchner. 2000. Significant increase in antigastric autoantibodies in a long-term follow-up study of *H. pylori* gastritis. *Virchows Arch.* **437**: 37-45.

Wadstrom, T. 1995. An update on *Helicobacter pylori*. *Current Opin. Gastroenterol.* **11**: 69-75.

Wagner, S., W. Beil, U.E.H. Mai, C. Bokemeyer, and M.P. Manns. 1994. Interaction between *Helicobacter pylori* and human gastric epithelial cells in culture: Effect of antiulcer drugs. *Pharmacol.* **49**: 226-237.

Wang, J., E.C. Brooks, K.B. Bamford, T.L. Denning, J. Pappo and P.B. Ernst. 2001. Negative selection of T cells by *Helicobacter pylori* as a model for bacterial strain selection by immune evasion. *J. Immunol.* **167**: 926-934.

Watanabe, K., K. Murakami, R. Sato, T. Okimoto, K. Maeda, M. Nasu, A. Nishizono, and T. Fujioka. 2004. CTLA-4 blockade inhibits induction of *Helicobacter pylori*-associated gastritis in mice. *Clin. Exp. Immunol.* **135**: 29-34.

Watanabe, T., M. Tada, H. Nagai, S. Sasaki, and M. Nakao. 1998. *Helicobacter pylori* infection induces gastric cancer in Mongolian gerbils. *Gastroenterol.* **115**: 642-648.

Watanbe, S., A. Takagi, Y. Koga, S. Kamiya, and T. Miwa. 2000. *Helicobacter pylori* induces apoptosis in gastric epithelial cells through inducible nitric oxide. *J. Gastroenterol. Hepatol.* **15**: 168-174.

Weeks, D.L., S. Eskandara, D.R. Scott, and G. Sachs. 2000. A H^+-gated urea channel: the link between *Helicobacter pylori* urease and gastric colonization. *Science* 287: 482-485.

Wen. Y., E.A. Marcus, U. Matrubutham, M.A. Gleeson, D.R. Scott, and G. Sachs. 2003. Acid-adaptive genes of *Helicobacter pylori*. *Infect. Immun.* **71**: 5921-5939

Wendling, D., J.M. Didier, D.A. Vuitton. 1991. The phagocyte oxidative metabolism function in alkylosing spondylitis. *Rheumatol.* **11**: 187-189.

Werdmuller, B.F.M., T.B.M.M. van der Putten, T.G. Balk, C.B.H.W. Lamers, and R.J.L.F. Loffeld. 2000. Clinical presentation of *Helicobacter pylori*-positive and – negative functional dyspepsia. *J. Gastroenterol. Hepatol.* **15**: 498-502.

Westblom, T.U., and P. Unge. 1992. Drug resistance of *Helicobacter pylori*: memorandum from a meeting at the 6th international workshop on Campylobacter, Helicobacter and related organisms. *J. Infect. Dis.* **165**: 974-975.

Westblom, T.U., E. Madan, and B.R. Midkiff. 1991. Egg yolk emulsion agar, new medium for the cultivation of *Helicobacter pylori*. *J. Clin. Microbiol.* **29**: 819-821.

Westblom, T.U., S. Phadnis, W. Langenberg, K. Yoneda, E. Madan, and B.R. Midkiff. 1992. Catalase negative mutants of *Helicobacter pylori*. *Eur. J. Clin. Microbiol. Infect. Dis.* **11**: 522-526.

Weston, A.P., A.S. Badr, M. Topalovski, R. Cherian, A. Dixon, and R.S. Hussanein. 2000. Prospective evaluation of the prevalence of gastric *Helicobacter pylori* infection in patients with GERD, Barrett's esophagus, Barrett's dysplasia, and Barrett's adenocarcinoma. *Am. J. Gastroenterol.* **95**: 387-394.

Westphal. O., and K. Jann. 1965. Extraction with phenol-water and further applications of the procedure. p. 83-90. In Methods in carbohydrate chemistry.eds.R.L. Whistler. Academic press.

Williams, R.R., P.D. Sorlie, M. Feinleib, P.M. McNamara, W.B. Kanna, and T.R. Dawber. 1981. Cancer incidence by levels of cholesterol. *JAMA* **245**: 247-252.

Wirth, H.P., M. Yang, M. Karita, and M.J. Blaser. 1996. Expression of human cell surface glycoconjugates Lewis X and Lewis Y by *Helicobacter pylori* isolates is related to cagA status. *Infect. Immun.* **64**: 4598-4605.

Wirth, H.P., M. Yang, R.M. Peek, J. Hook-Nikanne, M. Fried, and M.J. Blaser. 1999. Phenotypic diversity in Lewis expression of *Helicobacter pylori* isolates from the same host. *J. Lab. Clin. Med.* **133**: 488-500.

Wirth, H.P., M.H. Beins, M. Yang, K.T. Tham, and M.J. Blaser. 1998. Experimental infection of Mongolian gerbils with wild type and mutant *Helicobacter pylori* strains. *Infect. Immun.* **66**: 4856-4866.

Witteman, E.M., M.L. Verhulst, R.W. deKoning, W.P. Hopman, and J.B. Jansen. 1994. Basal serum gastrin concentrations before and after eradication of *Helicobacter pylori* infection measured by sequence specific radioimmunoassays. Ailment. *Pharmacol. Ther.* **8**: 515-519.

Wolle, K., A. Leodolter, P. Malfertheiner, and W. Konig. 2002. Antibiotic susceptibility of *Helicobacter pylori* in Germany: stable primary resistance from 1995 to 2000. *J. Med. Microbiol.* **51**: 705-709.

World Health Organization. 1966. WHO Technical series. Report No. **35**: 953.

Wotherspoon, A.C., C. Donglioni, T.C. Diss, L. Pan, A. Moschini, M. de Boni, and P.G. Isaacson. 1993. Regression of primary low-grade B-cell gastric lymphoma of mucosa associated lymphoid tissue type after eradication of *Helicobacter pylori*. *Lancet* **342**: 575-577.

Wu, A.H., J.E. Crabtree, L. Berbstein, P. Hawtin, M. Cockburn, C.C. Tseng, and D. Forman. 2003. Role of *Helicobacter pylori* CagA$^+$ strains and risk of adenocarcinoma of the stomach and esophagus. *Int. J. Cancer.* **103**: 815-821.

Wu, M.S., C.W. Lee, C.T.Shun, H.P. Wang, W.J. Lee, J.C. Sheu, and J.T. Lin. 1998. Clinicopathological significance of altered loci of replication error and microsatellite instability-associated mutations in gastric Cancer. *Cancer Res.* **58**: 1494-1497.

Wyle, F.A., A. Tarnawsky, D. Schulman, and W. Dabros. 1990. Evidence of gastric cell invasion by *C. pylori*: an ultrastructural study. *J. Clin. Gastroenterol.* **12**: S92-S98.

Xia, H., C.Y. Keane, S. Beattie, and C.A. O'Morain. 1994. Standardization of disk diffusion test and its clinical significance for susceptibility testing of metronidazole against *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **38**: 2357-2361.

Xiang, H.H., M.M. Jamshid, S. Kalntar, N.J. Talley, J.M. Wyatt, S. Adams, K. Cheung, and H.M. Mitchell. 2000. Antral type mucosa in the gastric incisura, body and fundus (Antralization): A link between *Helicobacter pylori* infection and intestinal metaplasia. American *J. Gastroenterol.* **95**: 114-121.

Yakabi, K., S. Ro, R. Okazaki, J. Shiojima, K. Tsuda, H. Mimura, H. Tomono, and T. Nakamura. 2000. Water extract of *Helicobacter pylori* stimulates interleukin-8 secretion by a human gastric epithelial cell line (JR-St) through protein tyrosine phosphorylation. *J. Gastroenterol. Hepatol.* **15**: 263-270.

Yamaguchi, H., T. Osaki, H. Taguchi, T. Hanawa, T. Yamamoto, and S. Kamiya. 1996. Flow cytometric analysis of the heat shock protein 60 expressed on the cell surface of *Helicobacter pylori*. *J. Med. Microbiol.* **45**: 270-277.

Yamaoka, Y., H.M. Malaty, M.S. Osato and D.Y. Graham. 2000. Conservation of *Helicobacter pylori* genotypes in different ethnic groups in Houston, Texas. *J. Infect. Dis.* **181**: 2083-2086.

Yamaoka, Y., S. Kikuchi, H.M.T. El-Zimaity, O. Gutierrez, M.S. Osato, and D.Y. Graham. 2002. Importance of *Helicobacter pylori* oipA in clinical presentation, gastric inflammation, and mucosal interleukin-8 production. *Gastroenterol.* **123**: 414-424.

Yang, J.C., T.H. Wang, H.J. Wang, C.H. Kook, J.T. Wang, and W.C. Wang. 1997. Genetic analysis of the cytotoxin-associated gene and the vacuolating toxin gene in *Helicobacter pylori* strains isolated from Taiwanese patients. *Am. J. Gastroenterol.* **92**: 1316-1321.

Ye, D., D.C. Willhite, and S.R. Blanke. 1999. Identification of minimal intracellular vacuolating domain of the *Helicobacter pylori* vacuolating toxin. *J. Biol. Chem.* **274**: 9277-9282.

Yokota, K., Y. Hirai, M. Haque, S. Hayashi, H. Isogai, T. Sugiyama, E. Nagamachi, Y. Tsukada, N. Fujii, and K. Oguma. 1994. Heat shock proteins produced by *Helicobacter pylori*. *Microbiol. Immunol.* **38**: 403-405.

Yokota, S.I., K.I. Amano, S. Hayashi, and N. Fujii. 1997. Low antigenicity of the polysaccharide region of *Helicobacter pylori* lipopolysaccharides derived from tumours of patients with gastric *Cancer*. *Infect. Immun.* **65**: 3509-3512.

Yokota, S.I., K.I. Amano, Y. Shibata, M. Nakajima, M. Suzuki, S. Hayashi, N. Fujii, and T. Yokochi. 2000. Two distinct antigenic types of the polysaccharide chains of *Helicobacter pylori* lipopolysaccharides characterized by reactivity with sera from humans with natural infection. *Infect. Immun.* **68**: 151-159.

Yoshida, N., D.N. Granger, D.J. Evans, Jr., D.G. Evans, D.Y. Graham, D.C. Anderson, R.E. Wolf, and P.R. Kvietys. 1993. Mechanisms involved in *Helicobacter pylori* induced inflammation. Gastroenterol. **105**: 1431-1440.

Yoshiyama, H. and T. Nakazawa. 2000. Unique mechanism of *Helicobacter pylori* for colonizing the gastric mucus. *Microbes and Infection* **2**: 55-60.

Young, G.O., N. Stemmet, A. Lastovica, E.L. van der Merwe, J.A. Louw, I.M. Modlin, and I.N. Marks. 1992. *Helicobacter pylori* lipopolysaccharide stimulates gastric mucosal pepsinogen secretion. *Ailment. Pharmacol. Ther.* **6**: 169-177.

Young, K.A., Y. Akyon, D.S. Rampton, S.G.R.G. Barton, R.P. Allaker, J.M. Hardie, and R.A. Feldman. 2000. Quantitative culture of *Helicobacter pylori* from gastric juice: the potential for transmission. *J. Med. Microbiol.* **49**: 343-347.

Yount, W.J., M.M. Domer, H.G. Kunkel, and E.A. Kabat. 1968. Studies on human antibodies. *J. Exp. Med.* **127**: 633-646.

Zheng, P.Y., J. Hua, K.G. Yeoh, and B. Ho. 2000. Association of peptic ulcer with increased expression of Lewis antigens but not cagA, ice A, and vacA in *Helicobacter pylori* isolates in an Asian population. *Gut* **47**: 18-22.

Zito, F., A. DiCastelnuovo, A. D'Orazio, R. Negrini, D. DeLucia, M.B. Donati, L. Iacoviello. 1999. *Helicobacter pylori* infection and the risk of myocardial infarction: role of fibrinogen and its genetic control. *Thromb. Haemost.* **82**: 14-18.