Genetic and environmental factors associated with *Helicobacter pylori* and peptic ulcer disease

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Ph. D.

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1997

Declaration

I declare here that the work for this thesis was carried out by myself or under my direct supervision.

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I dedicate this work to my mother Nuria, my wife Kamila, my son Abdulrahman, my aunt Zohra, my brothers, my sisters and the memory of my father Mohamed,

Acknowledgements

During my study in University of Edinburgh I received the help and goodwill of many people. With great pleasure I acknowledge my gratitude to my supervisors: Dr. Caroline Blackwell and Prof. Donald Weir for enthusiasm, encouragement and dedicated guidance. It is thanks to the long hours of patient discussions they have spent with me which created a good atmosphere for the research to be accomplished.

I would like to thank my colleagues in the Infection and Immunity laboratory for their unreserved help and advice. In particular, many thanks to Mike Kerr, Valerie James and Malcolm Baldock for assisting me. Thanks to Dr. Ian Poxton for his advice and discussions regarding outer membrane proteins and gel electrophoresis techniques.

I would like also to thanks the staff of Gastroenterology unit in Western General Hospital in particular Dr. KR Palmer and Dr. W Luman for collection of gastric biopsies and blood samples. Thanks also to Dr. Mary Hanson and Sheila Holgate for helping during culturing of *Helicobacter pylori* in the Medical Microbiology laboratory in Western General Hospital.

I am grateful to the Ministry of Education in Libya and El-Fatah University which funded the research and thanks also to Dr. Salem Al-Zaroug and Dr. Ibrahim Al-Zobidy for encouragement and support.

Finally, I express gratitude to my wife Kamila and my family at home for all their support, encouragement over the years.

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Abbreviations

BEC	Buccal epithelial cells
BI	Binding index
CLO	*Campylobacter* like Organism
DU	Duodenal ulcer
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
Gal	Galactose
GalNac	N-acetylgalactosamine
GlcNac	N-acetylglucosamine
HSB	High salt buffer
IHD	Ischaemic heart disease
IIF	Indirect immunofluorescence
IL	Interleukin
Kato III	Gastric adenocarcinoma cell line
Le^a	$Lewis^a$
Le^b	$Lewis^b$
Le^x	$Lewis^x$
LPS	Lipopolysaccharides
NO	Nitric oxide
OD	Optical density
OMP	Outer membrane protein

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PU	Peptic ulcer
SDS-PAGE	Sodium dodecyl sulphte-polyacrylamide gel electrophoresis
TNF	Tumour necrosis factor

ABSTRACT

*Helicobacter pylori in*fection is associated with gastritis and peptic ulceration. Epidemiological studies indicated that individuals of blood group O or non-secretors of their ABO blood group antigens are over represented among patients with peptic ulcers.

The aims of the study were: 1) to assess blood group and secretor status of local patients with peptic ulcer disease; 2) to determine if *H. pylori* binds to blood group antigens and if environmental factors such as fasting or smoking affect the binding; 3) to isolate bacterial adhesins that bind to the blood group antigens; 4) to determine if there are differences in the immune or inflammatory responses to *H. pylori* associated with ABO blood group or secretor status.

There was a higher proportion of group O and non-secretors in the local patient group. In studies in which flow cytometry was used to assess bacterial binding to buccal epithelial cells or the gastric adenocarcinoma cell line (Kato III), binding of *H. pylori* was inhibited by pretreatment of the cells with monoclonal antibodies against Lewis^a (Le^a), Lewis^b (Le^b) and H type 2 (the antigen of blood group O). Binding indices of *H. pylori* to buccal epithelial cells correlated with binding indices for H type 2 and Le^b but not with Le^a, suggesting the terminal fucose moieties of H type 2 and Lewis^b are important epitopes for bacterial binding.

Binding of *H. pylori* to buccal epithelial cells from smokers was significantly higher than to cells from non-smokers among patients referred to the gastroscopy clinic. In other experiments with cells from healthy volunteers, binding of *H. pylori* to cells from smokers was higher than to cells of non-smokers but the differences were not significant. Because each patient had fasted for approximately 12 hours before the cells were collected at the clinic, the effects of fasting and smoking were

examined with buccal cells from 15 pairs of smokers and non-smokers when fasting during Ramadan and after the fast when the donors were eating and drinking normally. In this group, binding of *H. pylori* and expression of H type 2 were significantly lower among the smokers. For both the patients and healthy groups, the levels of H type 2 expressed on buccal epithelial cells correlated with binding of *H. pylori,* and H type 2 is expressed in significantly greeter levels on cells of group O.

A protein of approximately 61 kDa was obtained by affinity adsorption with synthetic Lewis^a, Lewis^b or H type 2 antigens conjugated to Synsorb beads. The amount of material obtained from the Synsorb eluates reflected the amount of the biotinylated blood group antigens bound to the bacteria assessed by whole cell ELISA or dot blot; more H type 2 bound to whole cells of *H. pylori*, its outer membrane proteins or the 61 kDa adhesin than Le^b while Le^a bound the least effectively. The studies of inflammatory mediators released from human buffy coats in response to *H. pylori* or the purified adhesin showed that blood group O individuals produced significantly higher levels of IL-6, TNF and nitric oxide.

Higher levels of colonization by *H. pylori* are significantly associated with duodenal ulceration. The greater binding to H type 2 might contribute to the excess of individuals of blood group O in the patient population. The greater binding of Le^b found in the mucus of secretors to most strains compared with Le^a might more effectively block some of the bacterial adhesins, thereby reducing density of colonisation among secretors. Differences in inflammatory responses to *H. pylori* need to be further investigated with reference to the associations of peptic ulceration with group O and gastric carcinoma with group A.

Chapter 1 General introduction

1- Historical background

It is over a century since Bizzozero first described in 1893 spiral microorganisms in the gastric mucosa of dogs. In the early years of this century, similar organisms were found in the gastric contents of patients with ulcerative carcinoma and other reports confirmed these findings, noting also the absence of these organisms in healthy people [reviewed by Owen, 1995].

Helicobacter pylori was identified first in Southampton by Steer in 1975. He reported spiral bacteria on gastric mucosa, deep in the mucus layer of patients with gastric ulceration [Steer and Colin-Jones, 1975]. The organism seen on the mucosa was a spiral bacterium and it is now assumed that the bacteria were probably *H. pylori* . Electron micrographs of spiral bacteria in large numbers on gastric epithelial cells were published by Steer in 1984 [Steer, 1984]. In 1983 Marshall and colleagues reported a histological association between a spiral bacterium, peptic ulcer and gastritis. The following year the organism was found to have culture properties similar to members of the *Campylobacter* group. The taxonomic classification evolved from the initial term *Campylobacter*-like, through *Campylobacter pyloridis*, finally in 1989 to *Helicobacter pylori* [Goodwin *et al.*, 1989].

1.1- *Helicobacter* genus

The culture of *H. pylori* stimulated interest in the microbiology of the human stomach. The result was that a number of related bacteria from other animals have been cultured and shown to belong to the same genus as *H. pylori* by 16S rRNA sequencing, DNA hybridisation, or use of genus specific probes. *Helicobacter* isolates have been found in a diverse group of animals species (Table 1.1)

Table 1.1 *Helicobacter* genus cultured from different animal species

Host	***Helicobacter* Species**	**Reference**
rat and mouse	*Helicobacter muridarum*	Lee *et al.*, 1992
cat and dog	*Helicobacter felis*	Paster *et al.*, 1991]
pigtailed macaque	*Helicobacter nemestrinae*	Bronsdon *et al.*, 1991
cheetah	*Helicobacter acinonyx*	Eaton *et al.*, 1991
ferret	*Helicobacter mustela*	Fox *et al.*, 1986

Two human pathogens associated with gastroenteritis were originally put in the *Campylobacter* genus but are now know as *Helicobacter cinaedi* and *Helicobacter fennelliae* [Vandamme *et al.*, 1991]. *Helicobacter mustela*, the second species to be cultured, is a natural inhabitant of the ferret stomach [Fox *et al.*, 1986]. It's morphology is different from other *Helicobacter* species being a short rod shaped organism; however, it shares the major common biochemical and physiological features of the *Helicobacter* gastric group.

Flexispira rappini has been found in human beings with gastroentritis and in the stomachs of beagle dogs [Henry *et al.*, 1987]. Although this organism shows some differences from *H. pylori,* it possesses urease and genotypically should be in the *Helicobacter* genus as *Helicobacter rappini* [Lee *et al.*, 1992; Vandamme *et al.*, 1991]. The most widespread of the spiral gastric bacteria that colonise the mucosa is the bacterium currently known as *Gastrospirillum hominis* [McNulty *et al.*, 1989]. This bacterium is found in very large numbers in the stomach of cats, dogs and cheetahs. The organism gets its name from being associated with gastritis in a small number of humans [Lee *et al.*, 1988]

1.2 Characteristics of *H. pylori*

H. pylori is a Gram-negative, unipolar, multiflagellate, spiral organism with bluntly rounded ends, measuring 0.5 to 1.0 μm in width and 2.5 to 4.0 μm in length [Mégraud, 1989; Goodwin and Armtrong, 1990]. The organism might occasionally contain bacteriophages [Schmid *et al.*, 1990]. *H. pylori* colonises gastric type mucosa and is found primarily in the antrum of the stomach. It is also seen in the duodenum within areas of gastric metaplasia and in the fundus [Steer, 1984; Caselli *et al.*, 1988].

The organism produces large amounts of urease which is present within and on the bacterial surface [Dick, 1990; Blaser *et al.*, 1990]. Urease cleaves urea to produce ammonia which creates an alkaline environment that aids survival of the bacteria in the acidic environment of the gastric lumen and mucosa [Goodwin *et al.*, 1986; Hazell and Lee, 1986]. Prolonged culture gives rise to the coccoidal forms, which also appear after exposure to oxygen [Jones and Curry, 1992] . It is assumed that these forms are indicative of a dormant state and assist the survival of the organism in the environment [Jones and Curry, 1992]. The coccoidal forms of *H. pylori* are formed when the outer envelope appears to separate and ceases to keep pace with cell growth leading to folding of the inner cytoplasmic rod [Jones and Curry, 1992]

1.3 - Classification

H. pylori strains closely resemble one another in morphology, structure, physiology and biochemical characteristics. None of the common biochemical tests performed in clinical microbiology laboratories can distinguish between *H. pylori* strains [reviewed by Blaser, 1994]. Various phenotypic characteristics and a range of techniques have been tested in an effort to find a basis for discriminating logically between isolates of *H. pylori.*

1.3.1 - Phenotyping methods

1.3.1.1 - Biotyping

H. pylori appears to be a homogeneous species [Mégraud, 1989]. Biotyping using preformed enzyme activity only provides a low degree of discrimination between isolates of *H. pylori.* Among 126 *H. pylori* isolates from human and animals (monkey, baboon and pig), there were four biotypes detected by API Zym system using five enzymes. Among the strains from 10 countries, there was one predominant group (85%), biotype II (bio-type 2). They all characteristically

produced alkaline phosphatase, acid phosphatase, leucine arylamidase and naphthol phosphohydrolase, but not esterase C4 or C8. The other three biotypes which were less frequently encountered were detected with similar frequencies 4% type I, 6% type III and 5% type IV [Owen and Desai, 1990]

1.3.1.2 - Serotyping

The carbohydrate portion of lipopolysaccharides (LPS) consists of a core oligosaccharide which is conserved among the members of a species and more variable polysaccharide side-chains. The polysaccharide side chain can be of either variable or uniform length units. There is marked diversity in the length and antigenicity of the polysaccharide side chains among *H. pylori* strains [Pérez-Pérez and Blaser, 1987].

Lior described five serogroups based on heat labile (flagellar) antigens among 87 isolates investigated. Subsequent data showed that most strains appear to belong to the same serogroup [Lior, 1991].

The antigenicity of *H. pylori* LPS has been investigated and the LPS antigens of *H. pylori* were sufficiently diverse for potential use as the basis of an O-antigen (heat-stable) serotyping system [Mills *et al.*, 1992].

1.3.1.3 - Haemagglutination

Haemagglutination of *H. pylori* isolates with erythrocytes from 11 different animal species resulted in 9 haemagglutination patterns [Wadstrom *et al.*, 1990]. In another study, strains were classified into 3 major HA classes by using haemagglutination inhibition. Haemagglutination of 31% of strains in class I was inhibited by different combinations of N-acetylneuraminlactose (NANLac), orosomucoid or fetuin but not by asialofetuin. Strains in class II (38%) exhibited a different receptor specificity binding to different combinations of NANLac, orosomucoid and fetuin as well as

asialofetuin. Strains in class III (31%) showed no inhibition by these materials [Lelwala-Guruge *et al.*, 1992].

1.3.1.4 - Outer membrane proteins

The direct molecular characterisation of patterns of certain outer membrane protein (OMP) classes provides a means of comparing and typing isolates of *H. pylori*. *H. pylori* strains have been found to contain six major OMP bands with molecular masses of approximately 23, 27, 46, 58, 61 and 66 kDa [Morgan *et al.*, 1991]. A group of closely related high molecular weight proteins which migrate between 120 and 140 kDa were not present in all strains [Pérez-Pérez and Blaser, 1987; Apel *et al.*, 1988]. The cytotoxin associated gene A (*cagA*) that encodes these proteins has been cloned [Tummuri *et al.*, 1993], and it has been shown that about 60% of *H. pylori* strains posses the gene. The *cagA* gene was the first *H. pylori* gene used to differentiate between strains. *H. pylori* OMPs differ between the strains and a typing scheme based on four bands 31, 70, 90, and 110 kDa detected 16 typing groups with a wide scatter of strains among the groups [Goodwin and Armstrong, 1990].

1.3.1.5 - Immunoblotting

Immunoblotting with a rabbit antiserum raised against the protein extract of a selected *H. pylori* strain produced nine types, each defined on the basis of the presence or absence of 11 protein bands [Burnie *et al.*, 1988]. An immunoblot fingerprinting technique showed that *H. pylori* had 4 - 6 major immunogenic polypeptides in the 48 - 84 kDa region. Differences in minor antigenic bands in the 84 - 180 kDa regions allowed differentiation between isolates [Clayton *et al.*, 1991].

1.3.1.6 - Protein profiles

Typing of *H. pylori* isolates based on analysis of whole cell protein fingerprints obtained from high resolution one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that virtually all the strains of *H. pylori* tested had complex but stable protein patterns of 35 to 40 discrete bands with molecular weights of 20 to 100 kDa [Costas *et al.*, 1991]. Reproducible quantitative and qualitative differences in the bands were used to distinguish between strains [Owen *et al.*, 1989]. Similar patterns were found only between isolates from the same individuals [Costas *et al.*, 1991].

1.3.2 - DNA-based typing methods

DNA based typing methods indicate there is considerable diversity among *H. pylori* isolates.

1.3.2.1 - Restriction endonuclease digest patterns

DNA digest patterns have been used widely to compare isolates of *H. pylori* from different individuals, to distinguish multiple isolate sets before and after treatment [Beji *et al.*, 1989; Simor *et al.*, 1990; Oudbier *et al.*, 1990; Owen *et al.*, 1990], to compare isolates from dental plaque and stomach [Shames *et al.*, 1989] and isolates from different individuals in the same family [Nwokolo *et al.*, 1992]. Digest patterns are highly complex with multiple band patterns. It is difficult to quantify similarities between strains because most strains from different individuals appear to have unique DNA digest patterns. One possible approach is to use the larger sized fragments of 10 to 20 kb to provide a signature pattern for high level discrimination and to subgroup the isolates [Owen *et al.*, 1990]. Another approach is to compare different isolates by pulsed field gel electrophoretic analysis of DNA digested with low frequency cutting enzymes such as those from *Nocardia otitidis caviarum* (*NotI*) and *Nocardia rubra* (*NruI*) [Taylor *et al.*, 1992].

1.3.2.2 - Southern blot hybridization analysis

Ribosomal RNA gene patterns (ribopatterns) provide a simpler yet highly discriminatory basis for strain identification. The pattern of bands reflects the location of restriction sites in and around the two copies of the small 16S and large 23S rRNA subunit genes [Owen, 1989]. Several investigators have used ribotyping to study pre- and post-treatment isolates of *H. pylori* [Tee *et al.*, 1992; Rautelin *et al.*, 1994]. The ribopatterns obtained with *Haemophilus aegypticus* (*HaeII*) and *Haemophilus influenzae* (*HindIII*) are highly discriminatory with distinctive profiles of five bands with sizes between 1 and 10 kb. Isolates from the same patients have similar genotypes, whereas strains from different individuals had different profiles except some family members in which similar patterns were observed [Bamford *et al.*, 1993; Nwokolo *et al.*, 1992].

1.4 - Epidemiology of infection

1.4.1 - Transmission of infection

The mode of transmission of *H. pylori* is unknown but the geographic and social patterns of *H. pylori* infection are consistent with human to human transmission via either the faecal - oral or oral - oral route. Evidence for person-to-person transmission include: infection among gastroenterologists performing upper endoscopy with presumed exposure to gastric secretions containing *H. pylori* [Mitchell *et al.*, 1989; Lin *et al.*, 1994]; transmission of *H. pylori* within families [Mitchell *et al.*, 1987; Malaty *et al.*, 1991; Lin *et al.*, 1991]; and high prevalence of *H. pylori* infection in institutionalised mentally handicapped patients compared with control populations [Lambert *et al.*, 1990; Berkowicz and Lee, 1987]. Direct evidence was obtained in studies in which volunteers who ingested the organism developed acute upper gastrointestinal symptoms and were colonized with *H. pylori* [Marshall I*et al.*, 1985; Morris and Nicholson, 1987].

1.4.1.1 - Faecal-oral transmission

There is a constant production of mucus and a rapid turnover of gastric mucosa in the stomach. *H. pylori* can be found in gastric juice and is constantly eliminated from the intestine. It can survive in faeces and can be excreted into the environment. Faeces or anything contaminated (*e.g.* water or food) could be the vehicle of transmission following the model of *Shigella* species or *Salmonella typhi* [summarised by Mégraud, 1995]

H. pylori has never been cultured from faecal specimens using the standard protocols, but large fresh stool samples suspended in a microaerobically maintained buffer were used to culture of *H. pylori* [Thomas *et al.*, 1992; Kelly *et al.*, 1994]. It has been also detected in faeces by the polymeriase chain reaction (PCR). An amplification product was demonstrated in the faeces of 26 of 29 patients [Mapstone *et al.*, 1993]. The prevalence of *H. pylori* infection has been directly related to the source of drinking water in Lima, Peru [Klein *et al.*, 1991] and to consumption of uncooked vegetables and uncooked shellfish in Chile [Hopkins *et al.*, 1993].

1.4.1.2 - Oral-oral transmission

Support for oral-oral transmission of *H. pylori* has also come from the detection of *H. pylori* DNA in the saliva, gastric juice and dental plaque of infected patients by PCR [Valentine *et al.*, 1991; Mapstone *et al.*, 1993; Banatvala *et al.*, 1993]. It is suggested that reflux of gastric juice containing *H. pylori* could reach the oral cavity and the bacteria could colonise the mouth, in particular the microaerophilic environment of the dental plaque. Saliva could be the vehicle of transmission [summarised by Mégraud, 1995].

1.4.2 - Age is a risk factor for infection

Epidemiological studies have relied upon serological evidence of a specific immune response to *H. pylori* antigens. The prevalence of *H. pylori* infection related to age in different communities shows an age-dependent increase and appears to fall into two groups. In the first group there is a high incidence of acquisition of infection during childhood (developing countries). In the second group, the prevalence of *H. pylori* infection increases with age (developed countries).

1.4.2.1 - Prevalence of *H. pylori* infection in developing countries

Most epidemiological studies on prevalence of *H. pylori* in different population used enzyme linked immunosorbent assays (ELISA) to detect antibodies to these bacteria, their outer membrane components or urease. In the Ivory Coast of Africa, 54% of individuals below the age of 9 years had antibodies to *H. pylori* and this rose to 70 - 80% throughout adult life [Mégraud *et al.*, 1989]. Serum samples from Vietnam showed that only 13.1% of children below the age of 9 years were antibody positive but the proportion rose to 43% between the age of 10 - 19 years and to 50 - 80% during adult life [Mégraud *et al.*, 1989]. In rural Thailand the prevalence of infection was 18% among children aged 5 - 9 years but reached 55% by 30 years and 75% by 50 years [Pérez-Pérez *et al.*, 1990]. A study from Belgium investigated the prevalence of *H. pylori* antibody in children between 2 and 14 years old. There was an increase in positivity from 5.4% in the age group 2 - 8 years to 13.4% in the age group 8 - 14 years [Blecker *et al.*, 1993].

The ELISA for immunoglobulin G antibody against the urease antigen of *H. pylori* was used for a clinical survey in Saudi Arabia. The prevalence of *H. pylori* infection increased rapidly with age from 40% in those aged 5 -10 years to more than 70% in those aged 20 years or more [Al-Moagel *et al.*, 1990]. In South Africa, there was an age-specific increase in the prevalence of *H. pylori* with 60% seropositive by the age of 10 years and 94% by the age of 30 years [Sathar *et al.*, 1994]

1.4.2.2 - Prevalence of *H. pylori* infection in developed countries

In developed countries, the presence of *H. pylori* infection seems to be low in children and increases gradually with age from 10% in young adults to reach a peak of 70% by the eighth decade [Rautelin and Kosunen, 1991]. Among asymptomatic healthy subjects in California, USA, the prevalence of *H. pylori* infection increased from 10% in those between the ages of 18 to 29 years to 47% in those between the ages of 60 to 69 years [Dooley *et al.*, 1989]. Two strains of *H. pylori* were used in a serological survey of the Algerian population in France. Results for 277 serum samples from blood banks and a children's clinic indicated 43% of the children below the age of 9 years had antibody to the bacteria. The prevalence of antibody positive individuals rose steadily during adult life reaching a peak of 92% between the ages of 40 to 49 years [Mégraud *et al.*, 1989].

Sera from health centres and emergency rooms in France examined by ELISA showed few children were infected before the age of 10 years but the proportion increased gradually to 36.7% in the sixth decade of life [Mégraud *et al.*, 1989]. Sera from blood donors in Finland showed the proportion of donors with IgG antibody against *H. pylori* increased from 10% between 18 to 25 years up to 60% in the donors in the age range of 56 to 65 years [Kosunen *et al.*, 1989]. In London, UK, the prevalence of IgG antibodies against *H. pylori* ranged from 9% in those aged less than 30 to 67% in those aged more than 70 years old [Mendall *et al.*, 1992].

The birth cohort pattern of peptic ulcer disease is found to be similar in all European countries, USA, Australia and Japan. In relation to gastric ulcers, the birth related risk involves all ages over 5 years, while in duodenal ulcer it does not start before the age of 15 years [Sonnenberg, 1995]. The link between *H. pylori* associated antral gastritis and duodenal ulcer disease is weaker in children than it is in adults

[Oderda *et al.*, 1991; Mitchell *et al.*, 1993]. Most Children with *H. pylori* infection do not seem to suffer from symptoms [Gormally *et al.*, 1995].

1.4.3 Socio-economic conditions

Apart from the well known effect of age on prevalence [Graham *et al.*, 1991], it is agreed that lower socio-economic status is associated with a higher prevalence of *H. pylori* infection. This relationship has been found world-wide: USA [Graham *et al.*, 1991; Smoat *et al.*, 1994]; Chile [Hopkins *et al.*, 1993]; Peru [Klein *et al.*, 1991]; UK [Sitas *et al.*, 1991]; Saudi Arabia [Al-Moagel *et al.*, 1990]; India [Katelaris *et al.*, 1993]; and South Africa [Louw *et al.*, 1993]. Vietnamese immigrants in Australia (who are usually in the highest socio-economic class) have a lower infection rate than Vietnamese blood donors of the same age in Ho Chi Minh city living under very difficult conditions [Mégraud *et al.*, 1989; Dwyer *et al.*, 1988]. In the US, among healthy children and young adults (age range 3 - 21 years) the rate of infection was assessed by the socio-economic status determined by family income. The rate of infection in families with low incomes was higher than families with high incomes [Fiedorek *et al.*, 1990]. Another study found living conditions during childhood (*e.g.*, absence of a fixed hot water supply and domestic crowding) were independent risk factors for *H. pylori* infection [Mendall *et al.*, 1992].

Several studies make clear that socio-economic status is closely linked to education level and provide an overall measure of environmental factors such as the general level of hygiene, water supply, sanitation and crowding in the household [Graham *et al.*, 1991; Smoat *et al.*, 1994; Al-Moagel *et al.*, 1990; Katelaris *et al.*, 1993; Louw *et al.*, 1993]. One of the largest studies on the importance of socio-economic status was the Eurogast study, in which 3194 asymptomatic subjects from 17 different populations in 13 countries were tested by serology for *H. pylori* infection. The incidence of seropositive subjects was 34% in those with tertiary education, 47% in

those with secondary education and 62% in those who received only primary education [Eurogast study Group, 1993].

1.4.4 Smoking

Cigarette smoking is a risk factor for the development, maintenance and recurrence of peptic ulcer disease [Freston, 1989]. There is a positive correlation between the number of cigarettes smoked and peptic ulceration, especially for duodenal ulcers (DU) [Friedman *et al.*, 1974; Harrison *et al.*, 1979; McCarthy, 1984]. Smoking is associated with reduction in the healing rate of gastric and duodenal ulcers [Doll *et al.*, 1958; Korman *et al.*, 1981] and with an increase in recurrence of duodenal ulcers [Sontag *et al.*, 1984; Korman *et al.*, 1983]. Current cigarette smokers had a higher rate of infection than non-smokers and ex-smokers [Bateson, 1993]. Martin and colleagues found smoking increases the risk of DU formation in subjects who are infected with *H. pylori* [Martin *et al.*, 1989]. Studies of small numbers of patients with gastric or duodenal ulcers suggest that smoking is unlikely to promote ulcer formation in the absence of *H. pylori* [George *et al.*, 1990; Hui *et al.*, 1986].

The major risk factor for *H. pylori* infection seems to be the socio-economic status of the child's family. Children and adult smokers can form a family circle, young women and their partners who are less well educated and less affluent are most likely to smoke [Charlton, 1996] and smoking rates are increased among poor people and less educated people [Valentich, 1994].

1.4.5 ABO blood group and secretor status

The first genetic markers for susceptibility to peptic ulcers were noted in the 1950's, well before the observation linking *H. pylori* infection to the condition. Individuals of blood group O have a 30 - 40% higher incidence of duodenal ulcer than those of the other blood groups. Non-secretors, individuals who do not have the water

soluble form of their ABO blood group antigens in body fluids, are 40 - 50% more likely to develop a duodenal ulcer than secretors [Aird *et al.*, 1954; McConnell, 1980]. It is known that most patients with duodenal ulcers are colonised with *H. pylori* [Tytgat and Rauws, 1990]. Several studies found no association between serological evidence of *H. pylori* infection and ABO blood group [Hook-Nikanne *et al.*, 1990; Loffeld and Stobberingh, 1991]. Mentis and colleagues reported similar negative findings for ABO blood group and identification of *H. pylori* by culture or microscopy; but they found an association for both gastric and duodenal ulcers with blood group O [Mentis *et al.*, 1991].

Another study showed that non-secretion is a significant risk factor for gastroduodenal disease but is not associated with the presence of *H. pylori* in the antral biopsy of patients with symptoms of dyspepsia in the age range 19 to 70 years [Dickey *et al.*, 1993]. A clear correlation was found between ABO blood group and *H. pylori* infection among patients with rheumatoid arthritis compared with an age matched group 50 - 66 years. Significantly more patients of blood group O, all of whom were secretors of ABH substances, had *H. pylori* in the antral mucosa compared with patients of the other blood groups. It was concluded that blood group O individuals run a higher risk of developing a chronic *H. pylori* infection [Henriksson *et al*, 1993]. Further studies on the role of ABO blood group and secretor status in relation to disease rather than infection are required.

1.5 Pathogenic mechanisms of *H. pylori*

H. pylori is found in an indent of the apical membrane of mucus cells [Lee *et al.*, 1985; Steer, 1985; Buck *et al.*, 1986; Chen *et al.*, 1986; Bode *et al.*, 1988] or the luminal surface of mucus secreting cells within gastric pits. It does not invade the tissues [Jones *et al.*, 1984; Rollason *et al.*, 1984; Tricottet *et al.*, 1986; Blaser, 1987]. Some of the bacteria are found within the mucus layer or, more usually, between the

mucus layer and the underlying gastric epithelium [Hazell *et al.*, 1986]. The cell wall of *H. pylori* contains a lectin [Evans *et al.*, 1988] which selectively binds the organism to the mucus cell layer and the epithelial cell membrane. Lectins allow *H. pylori* to attach tightly to gastric epithelial cells [Slomiany *et al.*, 1989]. After attaching to the epithelial cells, *H. pylori* infection leads to polymerisation of actin below the epithelial cell membrane and generation of a cell structure called "attachment pedestals" by which it is attached to the plasma membrane [Goodwin *et al.*, 1986; Smoot *et al.*, 1989; Rosenberg *et al.*, 1991].

Virulence factors that allow *H. pylori* to colonize and survive in the hostile environment of the stomach which include spiral shape and motility, adaptive enzymes and proteins and bacterial adherence to gastric mucosal cells. Factors suggested to contribute directly to disruption of the gastric mucosal barrier include toxins, humoral responses, cytokines and cell mediated responses of the host.

1.5.1 Spiral shape and motility

Motility of *H. pylori* is important for penetration of the mucus layer. The curved structure of *H. pylori* and its flagella enable it to penetrate the gastric mucous layer and to colonize the underlying gastric epithelium [Lee *et al.*, 1993]. The most convincing evidence that motility is a virulence factor for *H. pylori* comes from studies in gnotobiotic piglets, the most motile strain of *H. pylori* which also produced a vacuolizing cytotoxin was also the most virulent strain [Eaton *et al.*, 1989].

1.5.2 Urease activity

H. pylori urease is a 500 - 600 kDa protein with a high substrate affinity and is probably localized in the outer membrane and periplasmic space of the bacteria [Hazell *et al.*, 1986; Lambert *et al.*, 1995]. The bacteria are protected from the

harmful effect of acid by production of urease which metabolises urea to ammonia [Hazell *et al.*, 1986; Marshall *et al.*, 1990; Mobley *et al.*, 1991]. The cytotoxic effect of ammonia [Blaser, 1990; Cover *et al.*, 1991; Mobley *et al.*, 1991; Triebling *et al.*, 1991] damages the surrounding cells at the tight junction between epithelial cells and causes hydrogen ions to diffuse back across the mucus layer [Hazell *et al.*, 1986]. Ammonia also increases antral pH and stimulates gastrin release and acid secretion [Morris and Nicholson, 1987; Levi *et al.*, 1989; Graham, 1991; Triebling *et al.*, 1991; Beardshall, 1992] leading to hyperacidity [Levi *et al.*, 1989].

The finding of hypergastrinaemia due to increased pH in the antrum has not been confirmed by other observers [Chittajallu *et al.*, 1991; Nujumi *et al.*, 1991; Chittajallu *et al.*, 1992]. It has been shown that ammonia can produce functional and morphological alteration of gastric epithelium *in vitro*, including reduction of transmucosal potential differences [Thomson *et al.*, 1989; Murakami *et al.*, 1988], enhanced back-diffusion of H+ to the epithelial cells [Hazell *et al.*, 1987] and increased bacterial adherence [Parsons *et al.*, 1984]. Ammonia may combine with neutrophil generated hypochlorous acid (HOCl) to produce cytotoxic products such as monochloramine and hydroxylamine [Mai *et al.*, 1992].

1.5.3 Other enzymes and proteins

Other toxic factors produced by *H. pylori* are ureolysin, mucin-degrading enzyme [Cover *et al.*, 1989], protease, lipases and phospholipases which damage the integrity of the gastric mucus layer. The mucus becomes more soluble and less hydrophobic at risk of further damage from acid and pepsin [Slomiany *et al.*, 1987; Kawano *et al.*, 1990]. Catalase inhibits the bactericidal effect of neutrophils [Babiour, 1978; Tytgat *et al.*, 1991]. Enzymes can also damage the epithelial cell membrane by breaking down lecithin [Slomiany *et al.*, 1987]. Several non-human *Helicobacter* species produce a partially heat labile, pronase inhibiting molecule

which inhibits acid secretion [Vargas *et al.*, 1991]. *H. pylori* has been reported to produce a similar protein capable of inhibiting acid secretion from parietal cells *in vitro* [Cave and Vargas, 1989].

1.5.4 Bacterial adherence

Adherence of *H. pylori* to the epithelial cell surface by adhesion pedestals has been demonstrated by electron microscopy [Hessey *et al.*, 1990]. Specificity of bacterial adherence implies interaction between bacterial adhesins and mucosal cell receptors. A variety of putative *H. pylori* adhesins have been identified, including a fibrillar haemagglutinin [Evans *et al.*, 1988], a protein that co-purifies with urease by size-exclusion chromatography [Fauchere and Blaser, 1990], a 31 kDa adhesin [Evans *et al.*, 1991], a 19.6 kDa pilus-like protein [Doig *et al.*, 1991], a 63 kDa molecule antigenically similar to exoenzyme S of *Pseudomonas* [Lingwood *et al.*, 1991] and an adhesin suggested to bind to the Lewis^b blood group antigen [Boren *et al.*, 1993].

1.5.5 Toxins

The cytotoxin produced by 50 to 60% of *H. pylori* isolates is a large molecule (>100 kDa) which induces non-lethal vacuolisation of a variety of cell lines [Tee *et al.*, 1995]. It is heat labile and protease sensitive [Leunk *et al.*, 1988; Cover *et al.*, 1990]. Strains of *H. pylori* from subjects with peptic ulcer are more likely to produce cytotoxin *in vitro* compared to those from subjects with non-ulcer dyspepsia [Eaton *et al.*, 1989; Figura *et al.*, 1989; Cover *et al.*, 1990]. Antibodies to a 120 - 128 kDa protein were present in 100% of ulcer patients compared with about 70% of infected controls [Crabtree *et al.*, 1991]. The gene encoding this protein has been cloned and called cytotoxin associated gene A (*Cag* A) [Tummuru *et al.*, 1993].

Moleculer analysis of vacuolating cytotoxin A (*vacA*) from multiple strains indicates that some regions are relatively conserved between groups of strains, whereas others

are highly divergent [Cover *et al.*, 1994; Atherton *et al.*, 1995]. Two strikingly divergent regions are the 50 base pairs encoding the second half of the signal sequence and a 700 base pair region near the middle of the gene. All strains examined possess one of two main types of signal sequence, s1 (which can be subtyped into s1a and s1b) and s2, and one of two types of mid-region, m1 or m2. Strains classed as *vacA* s1/m1 produce higher levels of vacuolating cytotoxin activity in *vitro* than s1/m2 [Atherton *et al.*, 1995]. Patients with peptic ulcer disease are more likely to be infected with strains of the *vacA* s1 genotype than with strains of s2 genotype and those with the vacA s1 genotype are closely associated with *cagA* positive status [Atherton *et al.*, 1995].

1.5.6 Humoral responses

The immunoglobulin classes and subclasses of circulating anti-*H. pylori* antibodies are consistent with a prolonged chronic mucosal infection. IgG and IgA are predominant and IgM antibodies rarely seen [Rathbone *et al.*, 1986]. Western blotting of mucosal antibodies show that different patients have different patterns of antibodies to the various antigens of *H. pylori* [Crabtree *et al.*, 1991]. Mucosal antibodies consistently detected a variety of surface antigens including the 28 kDa subunits of urease and 61 kDa subunit of urease, 56 kDa heat shock protein, 54 kDa flagellum protein and the 120 kDa and 87 kDa cytotoxin associated antigens [Newell *et al.*, 1994]. The 61 kDa protein is also reported to be a heat shock protein [Dunn *et al.*, 1992; Evans *et al.*, 1992].

H. pylori infection leads to the development of gastric autoimmunity [Negrini *et al.*, 1991] which might contribute to the persistence of chronic inflammatory cells in the gastric epithelium after eradication of *H. pylori* [Solcia *et al.*, 1994]. Binding of IgG antibodies to *H. pylori* should promote complement dependent phagocytosis and killing of bacteria by neutrophil polymorphs, this process occurs *in vitro* [Tosi and

Czinn, 1990]; but *H. pylori* are resistant to phagocytosis by neutrophils which could be due to an inhibitory effect of ammonium ions [Kist *et al.*, 1993].

Mucosal IgA does not normally clear *H. pylori* infection. It might inhibit bacterial functions, neutralise harmful bacterial products and diminish antigen uptake. Patients with hypogammaglobulinaemia show what happens if humoral responses are lacking. These individuals rapidly develop gastric atrophy and are 50 times more likely to develop gastric cancer [Hermaszewski and Webster, 1993]. Gastric atrophy in these cases may be due to excessive antigen uptake in the absence of mucosal antibodies [Crabtree and Wyatt, 1993].

1.5.7 Cytokines and cell mediated responses

Electron microscopy of the mucosal cells shows damage to the plasma membrane, vacuolation of the cytoplasm and ingested bacteria [Wyle *et al.*, 1990]. These cells also show some features of activated macrophages in that they express HLA antigens [Wee *et al.*, 1992] which present antigens to CD receptors on lymphocytes [Crabtree *et al.*, 1994]. A number of inflammatory mediators have been shown to be increased in *H. pylori* infection: interleukin-1 (IL-1); interleukin-6 (IL-6); interleukin-8 (IL-8) [Crabtree *et al.*, 1991; Crabtree *et al.*, 1993; Noach *et al.*, 1994]; tumour necrosis factor-α (TNF-α) [Crabtree *et al.*, 1991]; interferon-γ [Fan *et al.*, 1993]; and platelet activating factor (PAF) [Denizot *et al.*, 1990]. *H. pylori* induces gastric epithelial cells to increase expression of IL-8 which attracts neutrophil polymorphs and induces these and mononuclear cells to express cytokines including IL-8 itself [Crabtree *et al.*, 1993].

H. pylori also induces blood monocytes and polymorphs to release reactive oxygen species *in vitro* [Neilsen and Andersen, 1992] and in the gastric and duodenal mucosa of infected patients [Davies *et al.*, 1994]. Reactive oxygen species have a detrimental effect on the viscoelastic properties of gastric mucus [Grisham *et al.*,

1987] and can also cause tissue damage. Host tissues are normally protected against oxidative damage by antioxidant substances such as ascorbic acid. Ascorbic acid also diminishes the oxidative generation of harmful substances such as N-nitroso compounds [Mirvish, 1994]. *H. pylori* gastritis decreases the amount of ascorbic acid in gastric juice, which could increase the susceptibility of the mucosa to oxidative damage. Gastric juice ascorbate levels rise after eradication of *H. pylori* [Sobala *et al.*, 1993]. Free oxygen radicals have been implicated in carcinogenesis in other organs [Feig *et al.*, 1994].

1.5.8 Density of *H. pylori* colonization

H. pylori density is associated with *cagA* and *vacA* genotype; strains with these two genes colonise the mucosa more densely than *cagA* and *vacA* negative strains. Atherton and colleagues found a strong association between antral bacterial density and duodenal ulcer disease [Atherton *et al.*, 1996]. Alternatively, more dense infections associated with more antral inflammation and epithelial damage, might cause lower somatostatin expression [El-Omar *et al.*, 1995], contributing to the higher levels of gastrin and acid production [Kaneko *et al.*, 1992] which might predispose to duodenal ulceration.

1.5.9 Glycoconjugates on the Lipopolysaccharide of *H. pylori*

H. pylori LPS has low endotoxic activity, induces a low immunological response, and has been implicated in a variety of biological interactions [Moran, 1995]. These include: an inhibitory effect on mucus glycosylation; interference with mucosal integrity; inhibition of the interaction of mucin with its receptor [Pilotrowski *et al.*, 1993]; the stimulation of pepsinogen secretion; and a role in the mediation of adherence of the bacterium to laminin in the basement membrane [Valkonen *et al.*, 1994].

The O-antigen regions of the LPS purified from *H. pylori* contain partially fucosylated N-acetyllactosaminoglycan chains terminated by a Lewis type 2 immunodetrminant, either Lewis^x or Lewis^y [Aspinall and Monteiro, 1996; Aspinall *et al.*, 1996]. Immunoelectron microscopy and ELISA were used to demonstrate presence of Le^x in LPS but Le^a or Le^b were not identified by these methods [Sherburne and Taylor, 1995]. The expression of Le^x epitopes on the surface of the bacterium may play a role in the development of an autoimmune reaction against host cells expressing similar antigens which might contribute to disease [Aspinall *et al.*, 1996]

1.6 *H. pylori* associated disease

H. pylori infection is almost always associated with inflammation; however, peptic ulcer disease and gastric carcinoma occur in only a subset of individuals infected chronically with *H. pylori*. There is now mounting evidence that *H. pylori* plays a major etiological role in peptic ulcers and there are two different patterns of gastritis, one associated with duodenal ulceration and the other with gastric ulceration .

1.6.1 *H. pylori* and gastritis

It is now well established that *H. pylori* is the main etiological agent underlying chronic gastritis. Evidence to support this comes from both human and animal studies as well as therapeutic trials. Marshall ingested a culture of *H. pylori* and within seven days developed acute gastritis in which the organisms were identified in the biopsy [Marshall *et al.*, 1985]. A similar second experiment on a volunteer produced a picture of chronic gastritis in which hypochlorhydria continued for some weeks [Sobala *et al.*, 1991] . In 1984 Marshall and colleagues isolated *C. pylori* (*H. pylori*) from biopsy specimens taken from consenting patients presenting for gastroscopy. The bacteria were present in almost all patients with active chronic gastritis, duodenal ulcer, or gastric ulcer. They found a close association between

H. pylori and antral gastritis [Marshall and Warren, 1984]. In addition, the therapeutic trials in which patients with gastritis were treated with either placebo or a combination of nitrofuran and amoxycillin showed that simultaneous clearance of *H. pylori* and improvement of gastric histology was seen only in those patients treated with antibiotics [Moss and Calam, 1992].

1.6.2 *H. pylori* and duodenal ulcer

H. pylori infection is associated world-wide with duodenal ulcers; the infection was diagnosed in 94% of 1695 duodenal ulcer patients [reviewed by Kuipers *et al.*, 1995]. Duodenal ulceration is associated with chronic duodenitis, an observation supported by one study in which 82% of 219 patients followed up for symptomatic duodenitis developed one or more duodenal ulcers [Sircus, 1985]. A long term follow up study has shown that subjects with antral gastritis are 14 times more likely to develop a peptic ulcer than those with normal gastric mucosa [Sipponen *et al.*, 1990]. A common histological finding in chronic duodenitis is surface gastric metaplasia. It is thought to be a consequence of hyperacidity and independent of duodenitis or *H. pylori* colonization, but *H. pylori* can be detected in the duodenum only in the presence of gastric metaplasia and not in normal tissue [Steer, 1984; Johnston *et al.*, 1988].

Therapeutic evidence has supported a role for *H. pylori* in duodenal ulceration. Before *H. pylori* was formally identified, it was reported that remission periods in patients with duodenal ulcer were prolonged following tripotassium dicitratobismuthate (bismuth chelate) which is active against *H. pylori,* rather than an H2 antagonist alone. There is now a wealth of information showing that the relapse rate of duodenal ulceration is reduced by eradication of *H. pylori* [Moss and Calam, 1992]. The most important evidence linking *H. pylori* and ulcer disease is the finding that successful eradication of *H. pylori* speeds up ulcer healing [Marshall

et al., 1988; Labenz and Borsch, 1994], prevents duodenal and gastric ulcer relapse and reduces ulcer complications. Clearly, *H. pylori* eradication has marked effect on the natural history of peptic ulcer disease [reviewed by Rauws and Tytgat, 1995]. Eradication of *H. pylori,* defined as the inability to demonstrate the bacteria on antral biopsy one month after the initial clearance of the organism with antibacterial treatment, is associated with prolonged remission for healed duodenal ulcers [Coghlan *et al.*, 1987]

1.6.3 *H. pylori* and gastric ulcer

H. pylori infection was diagnosed in 86% of 1022 gastric ulcer patients [reviewed by Kuipers *et al.*, 1995]. The diagnosis of *H. pylori* infection in gastric ulcer patients is more problematic than in duodenal ulcer patients. Gastric ulcer disease is associated with a higher prevalence of atrophic gastritis, intestinal metaplasia and with a lower acid output. These conditions lead to a more diffuse spread of the bacteria throughout the stomach and to the lowering of colonization densities particularly in the antrum [Fiocca *et al.*, 1992; Louw *et al.*, 1993]. This increases the risk of false-negative results from biopsy. A study of 36 patients with atrophic gastritis found *H. pylori* in biopsy specimens from only 33%, but 86% had serological evidence of infection [Karnes *et al.*, 1991]. Other studies suggest that eradication of *H. pylori* reduces the relapse rate as well as enhances healing in patients with gastric ulcers [Graham *et al.*, 1992]

Gastric ulcers also result from use of non-steroidal anti-inflammatory drugs (NSAIDs). The prevalence of gastric erosions in patients on long term NSAID therapy is about 50%, but the prevalence of gastric ulcers is at least 15% [Larkai *et al.*, 1987; Silvos *et al.*, 1979]. The prevalence of *H. pylori* infection in patients taking NSAID has been reported to range from 22% to 63% [Loeb *et al.*, 1990; Maxton *et al.*, 1990]. Histological evidence of gastritis in patients taking NSAID is

related to *H. pylori* infection rather than use of NSAID [Shallcross *et al.*, 1990; Laine *et al.*, 1991].

1.6.4 *H. pylori* and gastric cancer

Between 60 and 100% of subjects with gastric adenocarcinoma are infected with *H. pylori* at diagnosis. It has been postulated that type B gastritis due to *H. pylori* leads to intestinal metaplasia, atrophic gastritis, achlorhydria and finally gastric cancer. *H. pylori* infection has been reported in 44 - 97% of subjects with atrophic gastritis, 59 - 93% with intestinal metaplasia and 87 - 100% with dysplasia [reviewed by Lambert *et al.*, 1995].

Dooley [1991] reported that *H. pylori* might not cause gastric carcinoma but gastritis induced by this organism could act as the productive soil upon which other environmental variables act. Forman *et al.* [1990; 1991] found that antibodies to the organism are present in the majority of gastric cancer patients. Geographic studies of gastric cancer and *H. pylori* reveal close epidemiological parallels between the two [Parsonnet, 1993] (Table 1.2).

In animal models of gastric carcinogenesis, *H. pylori* infection appears to predispose to the development of gastric cancer [Fox, 1994]. As non-infected individuals never develop carcinoma, the relationship between *H. pylori* and different steps in carcinogenesis requires further investigation.

Table 1.2 Prevalence of *H. pylori* infection (% +ve) among patients with gastric cancer and controls in different countries.

Country	**Cases**		**Controls**	
	n	**% +ve**	**n**	**% +ve**
USA	69	52	252	38
Finland	54	70	84	51
Korea	28	89	30	67
Sweden	112	80	103	61
Japan	29	83	58	67
Taiwan	148	62	92	62
Netherlands	116	77	116	78
Portugal	80	70	80	81
Greece	47	72	50	68
Japan	213	88	213	76

Table reproduced from Forman (1995)

1.7 Diagnosis of *H. pylori* infection

The diagnosis of *H. pylori* infection like any other bacterial infection, involves identifying the bacterium by microscopy, culture, serology and/or specific DNA testing and detection of inflammation. Detection methods for *H. pylori* can be categorised as direct or indirect. Histologic demonstration of the organism or its identification by microbiological means from cultured tissue constitute direct evidence of its presence. Indirect techniques rely on detecting a characteristic of the bacteria such as hydrolysis of urea or the response of the immune system to its presence (specific antibodies).

1.7.1 Direct methods based on gastric biopsies

H. pylori is almost always found in the antral region where it induces inflammation, in the fundus and sometimes in the duodenal bulb. At endoscopy the tissue sample should be taken from the antral region at approximately 2 cm from the pylorus. Sampling errors can be minimised by taking two antral biopsies and by obtaining an additional biopsy from the body or from the fundus of the stomach. The experience of several investigators indicates that two biopsy samples will substantially increase the sensitivity [Hazell *et al.*, 1987; Goodwin and Armstrong, 1990]. Some authors have recommended brushing the gastric surface rather than taking biopsies in order to study a larger area [Debongnie *et al.*, 1992].

1.7.1.1 Histological examination

For histological examination, biopsies should immediately be put into a fixative such as formaldehyde or Bouin's solution. The particular morphology and location of *H. pylori* facilitate diagnosis by microscopy. It can be identified with a standard haematoxylin and eosin stain; the bacteria appear rose coloured [Taylor *et al.*, 1987]. The Giemsa stain is a more consistent and less costly alternative [Gray *et al.*, 1986].

Other stains that have been proposed for *H. pylori* diagnosis include Brown-Hopps [Westblom *et al.*, 1988], cresyl fast violet [Burnett *et al.*, 1987], histological half-Gram [Trowell *et al.*, 1987; Britt *et al.*, 1990], carbol fuchsin [Rocha *et al.*, 1989] and modified Wright's stain [Butler, 1990]. Excellent results were obtained with the Warthin Starry stain, a technique in which the bacteria stain black on a yellow background, but the technique is time consuming [Warren, 1983].

1.7.1.2 Rapid urease test

Urease catalyses urea to ammonia and bicarbonate. This reaction causes an increase in the pH of the surrounding medium, which can be detected with a pH indicator and the presence of urease is signalled by a colour change. The first urease test described for detection of *H. pylori* employed Christensen's 2% urea broth, a standard microbiologic reagent used to identify urease producers [McNulty and Wise, 1985]. The great advantage of this test is that it can be performed in the endoscopy room as soon as the biopsy has been taken. The *Campylobacter* - like organism (CLO) test is the most widely employed commercially available modification of the urea broth with the same phenol red indicator as Chistensen's urea broth [Marshall *et al.*, 1987]. The biopsy is placed on a special agar which has been brought to room temperature. A colour change from yellow to red is recorded after 30 min and 2 hours. The sensitivity of this test is in the range of 90 to 95% [Borromeo *et al.*, 1987; Bornschein *et al.*, 1989].

1.7.1.3 Bacteriological examination

Biopsies for culture should be maintained at 4^{o}C in a container with saline solution to avoid drying. *H. pylori* culture suspensions in distilled water and physiologic saline have been shown to remain viable for days at 7^{o}C, but the organism rapidly loses viability at room temperature [Hartmann *et al.*, 1987; West and Millar, 1990]. If the delay between biopsy and testing is longer than 4 hours, a transport medium

such as 20% glucose solution [Goodwin *et al.*, 1985], thioglycolate broth, nutrient broth, *Brucella* broth [Coghlan *et al.*, 1987], Stuart's transport medium [Faoagali *et al.*, 1986] or Portagerm pylori (bioMerieux) should be used. In Gram stains of smears prepared by rubbing a biopsy specimen on a glass slide, the spiral and curved bacteria appear Gram negative and are usually found in large numbers in different zones of the slide. This is a quick, simple and inexpensive test with a sensitivity about 80% [Montgomery *et al.*, 1987].

Biopsy suspension or biopsy specimens are inoculated on to two fresh plates; the first non-selective medium such as 10% blood agar, and a second selective medium [Tee *et al.*, 1991]. Skirrow's medium which contains vancomycin, trimethroprim and amphotericin B has been used by many workers to isolate *H. pylori*. Isolation rates are higher for selective medium compared with non-selective media [Leunk *et al.*, 1988]. Goodwin and colleagues have used nalidixic acid with vancomycin and amphotericin B as selective agents [Goodwin *et al.*, 1985]; however, 14% of the strains are sensitive to nalidixic acid and 5% of strains are sensitive to polymyxin B or colistin [Dent and McNulty, 1988]. An alternative agent which inhibits pseudomonads and other bacteria flora is Dent's formula (vancomycin, cefsulodin, trimethroprim and amphotericin B) [Dent and McNulty, 1988].

The plates are incubated for 3 to 7 days at 37^{o}C in a microaerophilic atmosphere. The colonies are identified by gross morphology and biochemical characteristics such as urease, oxidase, catalase and γ-glutamyl transpeptidase [Mégraud *et al.*, 1985].

1.7.1.4 Polymerase chain reaction (PCR)

PCR has the potential for diagnosing infection even when the target organism is in a non-culturable state or no longer alive. The first reports of the use of PCR for diagnosis of *H. pylori* infection used primers that amplified a fragment of the

bacterial ribosomal 16S gene [Hoshina *et al.*, 1990; Engstrand *et al.*, 1991]. Other investigators have used primers based on the urease gene [Valentine *et al.*, 1991; Clayton *et al.*, 1992]. The choice of the urease gene as a target for amplification assures that all urease negative organism are unlikely to interfere with detection of *H. pylori*. To evaluate gastric biopsy specimens, Hammar and colleagues used sequences of primers corresponding to the *H. pylori* gene encoding a 26 kDa antigen that appears to be species specific [Hammar *et al.*, 1992].

1.7.2 Indirect methods

1.7.2.1 Serology

H. pylori is not an invasive bacterium, but it actively stimulates the immune system of its host by release of lipopolysaccharides and immunogenic proteins. An immune response accompanies the presence of the bacterium in 98% of cases [Glupczynski *et al.*, 1992]. Among patients with *H. pylori* present in gastric biopsy, antibody titres to pooled *H. pylori* whole cell antigen are higher for IgG and IgA than among biopsy negative patients. IgM antibodies do not appear to differ between *H. pylori* negative and positive individuals [Rathbone *et al.*, 1986]. ELISA has been found to be more sensitive in detection of *H. pylori* antibody than bacterial agglutination or complement fixation [Jones *et al.*, 1986]. Many companies have developed commercial kits using purified antigen. Quick tests have also been developed based either on ELISA (Flexisure, Helisal, Immunocomb, Quick vue) or agglutination (Pylori set dry, *H. pylori* Kit). The ELISA tests available are generally satisfactory with regard to sensitivity and specificity. IgG does not usually appear until several weeks after infection, and after eradication, decreases in the titre are not significant until the 6th month [Kosunen *et al.*, 1992].

1.7.2.2 Urea breath test

A breath test based on *H. pylori*'s efficient hydrolysis of urea is the labelled carbon breath test. In infected individuals, the urea is metabolised to ammonia and labelled bicarbonate, and excreted in the breath as labelled carbon dioxide. The labelled carbon excreted can then be quantified. The original description of this technique used the stable, naturally occurring isotope ^{13}C [Graham *et al.*, 1987]. There are also commercial kits available (BSIA, Inbiomed). Another breath test using ^{14}C has been devised [Debongnie *et al.*, 1991; Henze *et al.*, 1990; Marshall and Surveyor, 1988]. ^{14}C can be easily quantified but it cannot be used in some countries for legal reasons.

1.8 Treatment of *H. pylori* infection

1.8.1 Antimicrobial agents

There are many treatment regimens available but their efficacy varies from centre to centre and from country to country. *H. pylori* is a bacterial infection, so an antimicrobial agent seems a logical therapeutic choice. *H. pylori* is sensitive to a large number of antimicrobials *in vitro* but not *in vivo*. The failure of antimicrobials to act *in vivo* include the preferred site of the bacteria buried deep in gastric pits protected by the mucous layer as well as the acid milieu of the stomach interfering with the drug's ability to destroy *H. pylori*.

Triple therapy with bismuth, metronidazole and tetracycline or substituting amoxycillin for tetracycline results in the best eradication rate with the lowest degree of variability [Tytgat and Noach, 1994]. Regimens including bismuth are the most effective and the most consistently reproducible. The more compliant the patients the more effective is the treatment. Resistance to metronidazole can be a limiting factor but is not a contraindication to triple therapy. Seven days of tetracycline and metronidazole appears to be efficient [Johnston *et al.*, 1993]; longer periods, whilst resulting in more side effects, seem to allow resistant strains to be eliminated

[Burette *et al.*, 1992]. In a study of 100 patients treated with bismuth, tetracycline and metronidazole for 15 days, eradication rates of 93% were achieved and less than 3% had significant side effects requiring the medication to be terminated [Thijs *et al.*, 1993].

1.8.2 proton-pump inhibitors

The proton-pump inhibitor omeprazole has also been proposed as a suitable addition to *H. pylori* treatment. Omeprazole directly suppresses but does not eradicate *H. pylori* infection unless combined with an antibiotic such as amoxycillin [Bayerdorffer *et al.*, 1993]. The combination of omeprazole and amoxycillin showed promise in achieving *H. pylori* eradication [Unge *et al.*, 1989]. The proton-pump inhibitors omeprazole, lansoprazole and pantoprazole all have intrinsic *in vitro* antimicrobial activity, but suppression rather than eradication is observed with each [Iwahi *et al.*, 1991; Suerbaum *et al.*, 1992]. Omeprazole, tetracycline and metronidazole achieved an eradication rate of 98% [McCarthy *et al.*, 1993]. Rates approaching 98% have been achieved with omeprazole, clarithromycin and amoxycillin. Use of these antibiotics for only 1 week have achieved rates over 90% [Logan *et al.*, 1994].

1.8.3 Sucralfate

Sucralfate, an aluminium salt of sucrose octasulphate, is a clinically effective drug for the prevention of acute gastric mucosal lesions induced by a topical irritant such as ethanol [Tarnawski *et al.*, 1987] or aspirin [Konturek *et al.*, 1986] and for the prophylaxis and therapy of peptic ulcer [Koeltz and Halter, 1989; Marks *et al.*, 1989]. Sucralfate reduced *H. pylori* density and improved chronic antral gastritis associated with duodenal ulcer [Hui *et al.*, 1989].

Slomiany and colleagues reported that both components of sucralfate, sucrose-octasulphate and aluminium, inhibit both *H. pylori* adhesion to the gastric mucosal surface and *H. pylori* growth [Slomiany *et al.*, 1992]. Sucralfate alone for 4 weeks and sucralfate with amoxycillin for 2 weeks cleared *H. pylori* in 17% and 47% respectively and eradication 4 weeks after ending treatment in 0% and 40% of patients respectively. Thus sucralfate alone suppresses but does not eradicate *H. pylori* infection [Hu *et al.*, 1993]. Sucralfate combined with either amoxycillin and metronidazole or tetracycline and metronidazole produced high eradication rates [Wurzer *et al.*, 1994].

1.9 Blood group antigens and susceptibility to infectious agents

1.9.1 ABO Blood groups

The ABO blood group system was first described by Landsteiner and colleagues early in this century. Their work demonstrated that individuals could be segregated into distinct groups based on the presence or absence of substances in serum that could agglutinate erythrocytes isolated from individuals of other groups. This work quickly found practical application in blood transfusion and modification of the original agglutinating methods are still in use in pretransfusion blood typing and cross matching [Mollison *et al.*, 1987; Walker, 1990; Rossie *et al.*, 1991]. Four major blood group were identified (Table 1.3). The ABO and Lewis blood group antigens are found not only on red cells but can be found in many secretions and on numerous cells of the human body (Table 1.4). The antigenic structures that characterise the ABO blood group are widely distributed in many animals, some plants and many bacteria. Springer [1970] demonstrated that Gram-negative bacteria express substances which are similar to the A, B and H (the antigen of blood group O) antigens of human.

Table 1.3 Antigens and isohaemagglutinins of the ABO blood group system

Group	Antigens on red cells	Antibodies in serum
A	A, H	Anti-B
B	B, H	Anti-A
AB	A, B, H	None
O	H	Anti-A and Anti-B

H is the blood group O antigen and also present on the cells of other blood groups.

Table 1.4 Distribution of ABO and Lewis blood group antigens on cells and in body fluids of secretors and non-secretors

Secretors	**H, A or B**	**Le^a**	**Le^b**
Secretions	+	-/+	+
Cells	+	-/+	+
Non-secretors			
Secretions	-	+	-
Cells	+	+	-

1.9.2 Epidemiological studies on infectious diseases

The associations between ABO blood groups and secretor status with susceptibility to infectious disease were collected and analysed by Mourant *et al.* [1978] and more recently by Blackwell and colleagues [Blackwell *et al.*, 1989]. Table 1.5 summarises the associations of infectious diseases and ABO blood groups. The secretor status is based on detection of the water soluble glycoprotein form of ABO antigens in body fluids (secretors). These are absent in a minority of individuals in most populations (20 - 25%) (non-secretors). Epidemiological studies demonstrated increased susceptibility for non-secretors of ABH blood group antigen to many diseases due to bacteria or yeasts or asymptomatic carriage of some species. Studies of viral diseases indicated that secretors were over represented among these patients (Table 1.6).

1.9.3 Secretor status and Lewis blood group antigens

In 1946, Mourant found an antibody in the serum of Mrs HDG Lewis which agglutinated red cells of approximately 20% of normal blood group donors. The antigen was initially referred to as Lewis and later Lewis^a or Le^a [Mourant, 1946]. Two years later Andresen described an antigen thought to be coded by an allele of Le^a that was called Lewis^b or Le^b [Andresen, 1948]. It was shown that all donors with Le^a positive red cells were non-secretors of ABH antigen and all donors with Le^b positive red cells were secretors of their ABH antigens in saliva [Grubb, 1948]. The predicted proportion of these two phenotypes within a population is secretors 75 - 80% and non-secretors 20 - 25%. This ratio can vary widely in different ethnic groups and some geographically isolated populations [Mourant *et al.*, 1976].

Table 1.5 Associations between infectious diseases and ABO blood groups

Infection agent and disease	Blood group	References
Respiratory tract		
Influenza A	O and B	Potter, 1969
Influenza A_2	O	
M. tuberculosis	B	Viskum, 1975
Oral cavity		
Candida albicans	O	Burford-Mason *et al.*, 1988
	O	Aly *et al.*, 1991;1992
Periodontal disease	O and AB	Pradhan *et al.*, 1971
Gastrointestinal tract		
E. coli	B	Socha *et al.*, 1969
Salmonella and *E. coli*	B and AB	Robbinson *et al.*, 1971
Vibrio cholerae	O	Barua and Paguio, 1977
Peptic ulcers	O	Aird *et al.*, 1953
Urinary tract		
E. coli	B and AB	Socha *et al.*, 1969
	B and AB	Kinane *et al.*, 1982
Genitourinary tract		
N. gonorrhoeae	B	Foster and Labrum, 1976
Malaria	A	Gupta and Chowdhuri, 1980

Table reproduced and modified from Blackwell (1989)

Table 1.6 Associations between infectious diseases and secretor status

Infection agent and disease	Secretor status	References
Oral cavity		
C. albicans disease	Non-secretors	Aly *et al.*, 1991
C. albicans carriage	Non-secretors	Aly *et al.*, 1992
Caries	Non-secretors	Holbrook and Blackwell, 1989
Respiratory tract		
Strep. pyogenes (group A)	Non-secretors	Haverkorn and Goslings, 1969
Strep. pneumoniae	Non-secretors	Blackwell *et al.*, 1986
Neisseria meningitidis	Non-secretors	Blackwell *et al.*, 1986
H. influenzae (type b)	Non-secretors	Blackwell *et al.*, 1986
Echo virus	Secretors	Raza *et al.*, 1991
Influenza A and B	Secretors	Raza *et al.*, 1991
Respiratory Syncytial Virus	Secretors	Raza *et al.*, 1991
Rhinovirus	Secretors	Raza *et al.*, 1991
Gastrointestinal tract		
Vibrio cholerae	Non-secretors	Chaudhuri and Adhikary 1978
Peptic ulcers	Non-secretors	Clarke et al., 1956
Genitourinary tract		
HIV (heterosexual transmission)	Secretors	Blackwell *et al.*, 1991

Table reproduced and modified from Blackwell (1989)

The *Le* gene has been cloned and codes for an α1,3/4 fucosyltransferase that is expressed in exocrine secretions and transfers fucose to the subterminal βGlcNAc unit of precursor chains [Kukowska *et al.*, 1990; Prieels *et al.*, 1981] (Figure 1.1). The secretor gene has been cloned and codes for an α1,2 fucosyltransferase that transfers fucose to the terminal βGal unit of precursor chains. The secretor fucosyltransferase is expressed in salivary glands, where its presence is correlated with the presence of ABH blood group substances in saliva [reviewed by Henry *et al.*, 1995].

Le^a and Le^b antigens do not form part of the structure of the cell membrane and are not synthesised on the red cells but are present in the plasma and in the body fluids. In secretions, the Lewis determinants are carried on the same glycoprotein molecules as the ABH antigens [Watkins, 1974].

1.9.4 Structures of the ABH antigens

The ABH blood group molecules are synthesised by the sequential action of enzymes termed glycosyltransferases [Watkins, 1980; Lowe, 1991]. Each enzyme is encoded by a distinct genetic locus and catalyses the addition of a specific monosaccharides to a specific position on the oligosaccharide precursor, or acceptor molecule [Dawson, 1978]. Some transferases such as that coded for by the *Le* gene, can transfer the same monosaccharides to several different acceptors leading to the formation of Le^a or Le^b.

1.9.4.1 Precursor chains

A, B and H substances are found mainly in the cell membranes as glycolipids, and mainly in secretions as glycoproteins. There are 4 major types of these precursor molecules to which different monosaccharides are added by glycosyltransferases to produce the A, B and H antigens. Type 1 and type 2 precursors are, in turn, found

covalently attached to some proteins by linkage to asparagine residues (asparagine-linked oligosaccharides) and also found attached to membrane associated lipid molecules (Figure 1.1). Type 1 chains are found mainly in the secretions and type 2 chains are found on the red cells and on most other cells of the body except the brain [Oriol *et al.*, 1986]. Type 3 and Type 4 precursor chains have been identified but not studied extensively as type 1 and type 2 [Takasaki *et al.*, 1978; Donald, 1981] and their biosynthetic regulation and distribution is not yet fully understood [Clausen *et al.*, 1986]

1.9.4.2 ABH antigens

ABH precursor oligosaccharides are modified by the action of α(1,2) fucosyltransferases. These enzymes catalyse a transglycosylation reaction in which fucose is transferred from the substrate GDP-fucose to the second carbon atom of the galactose displayed at the non-reducing terminus of the oligosaccharide precursor (Figure 1.2). The disaccharide unit formed in this manner, Fucα(1,2)Galβ-, represents the H blood group [reviewed in Watkins, 1980]. The *H* gene codes for production of a fucosyltransferse enzyme which adds L-fucose to the C2 position of the terminal sugar residue of Type 2 chains to form H Type 2. The A allele at the ABO locus encodes an α1,3N-acetylgalactosaminyl transferase that utilises H molecules to form the blood group A molecule, whereas the B allele encodes an α1,3 galactosyltransferase that forms blood group B determinants. The O allele is a null allele that does not encode a polypeptides capable of modifying H active precursors [reviewed in Watkins, 1980] (Figure 1.2).

The Type 1 precursor chain is fucosylated by the product of the *Se* gene, an α1-2 fucosyltransferase to form H Type 1 [Watkins *et al.*, 1988]. H Type 1 acts as a substrate for A or B glycosyltransferases to give rise to their respective antigens.

Type 1 **Galβ1——3GlcNAcβ————R (Protein chain)**

Type 2 **Galβ1——4GlcNAcβ————R (Lipid chain)**

Type 3 **Galβ1——3GalNAcα————R (Serines or threonines)**

Type 4 **Galβ1——3GalNAcβ————R (Ceramide)**

Gal = D-galactose

GlcNAc = N-Acetylgucosamine

GalNAc = N-Acetylgalactosamine

Figure 1.1 Main precursor types of ABH active oligosaccharide

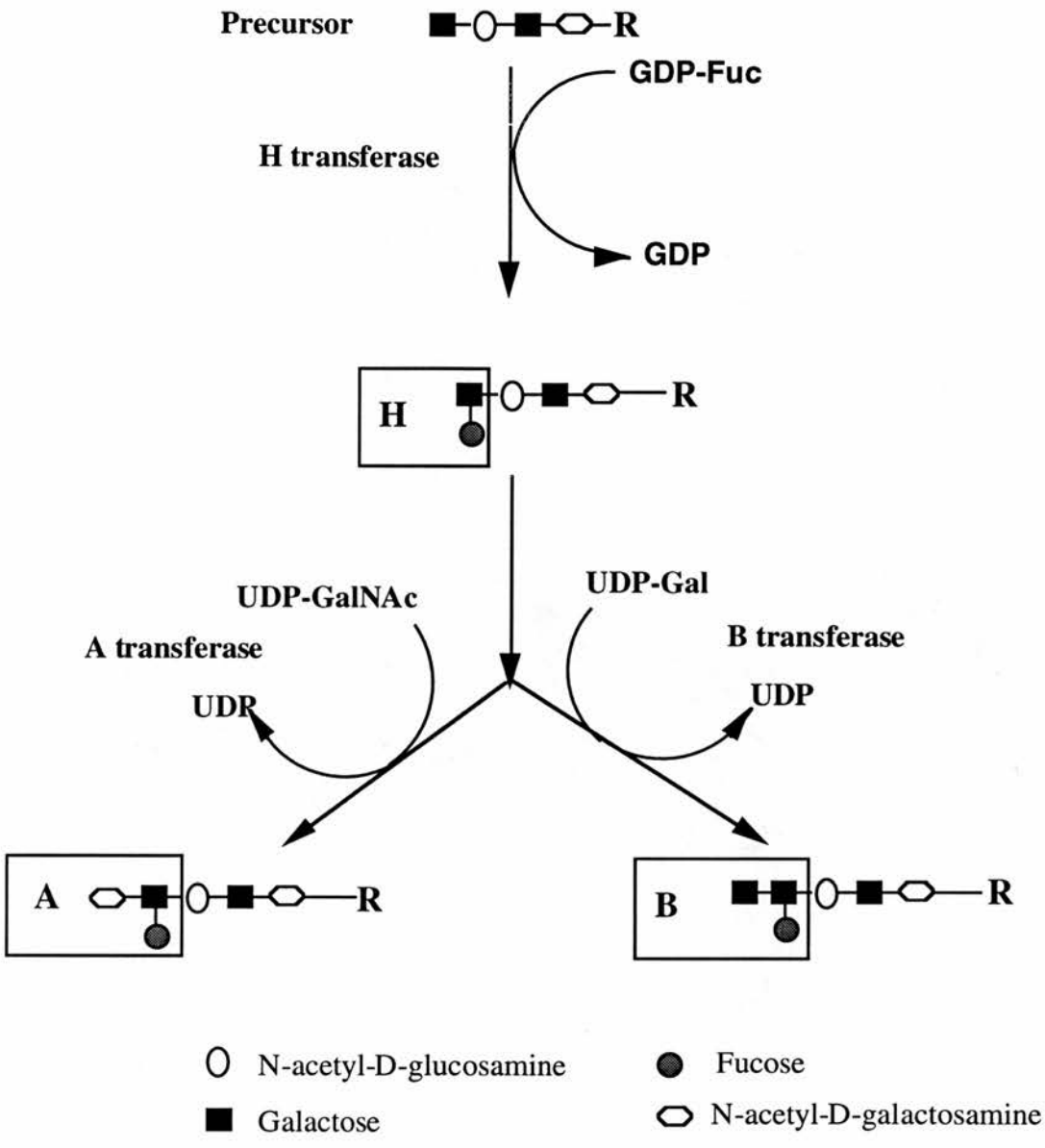


Figure 1.2 Production of A, B and H (O) antigens from precursor Type 1 and 2 chains. The blood group A transferase requires UDP-N-acetylgalactosamine as its sugar nucleotide substrate, whereas the blood group B transferase requires UDP-galactose. Type 3 and 4 A, B and H determinants are synthesized in an identical manner.

1.9.5 Interaction of the products of the *A*, *B*, *H*, *Se* and *Le* genes

Although there are two antigens, Le^a and Le^b, there is only one *Le* gene, which encodes an α1-3/4 fucosyltransferase. The Le^a antigens are the α(1-4) monofucosylated type 1 chain and Le^b antigens are α(1-2) and α(1-4) difucosylated type 1 chain [reviewed by Oriol *et al.*, 1986]. The enzyme coded by the secretor gene adds fucose to the terminal sugar of the type 1 precursor and that coded for by the Lewis gene adds fucose to the subterminal sugar of the type 1 precursor. If the secretor enzyme adds fucose first, the structure can act as a substrate for the Lewis enzyme to form Le^b antigen. If the Lewis enzyme adds fucose to the subterminal sugar first to form Le^a, the secretor gene can not use the Le^a as a substrate to form Le^b [Watkins *et al.*, 1988]. The presence of Lewis antigens in secretions and on cell surfaces depends on the expression of the *Le* gene and not on the *Se* gene. Individuals who do not express any Lewis determinants on their red blood cells or in secretions are known as Lewis-negative (Le^{a-b-})(Figure 1.3).

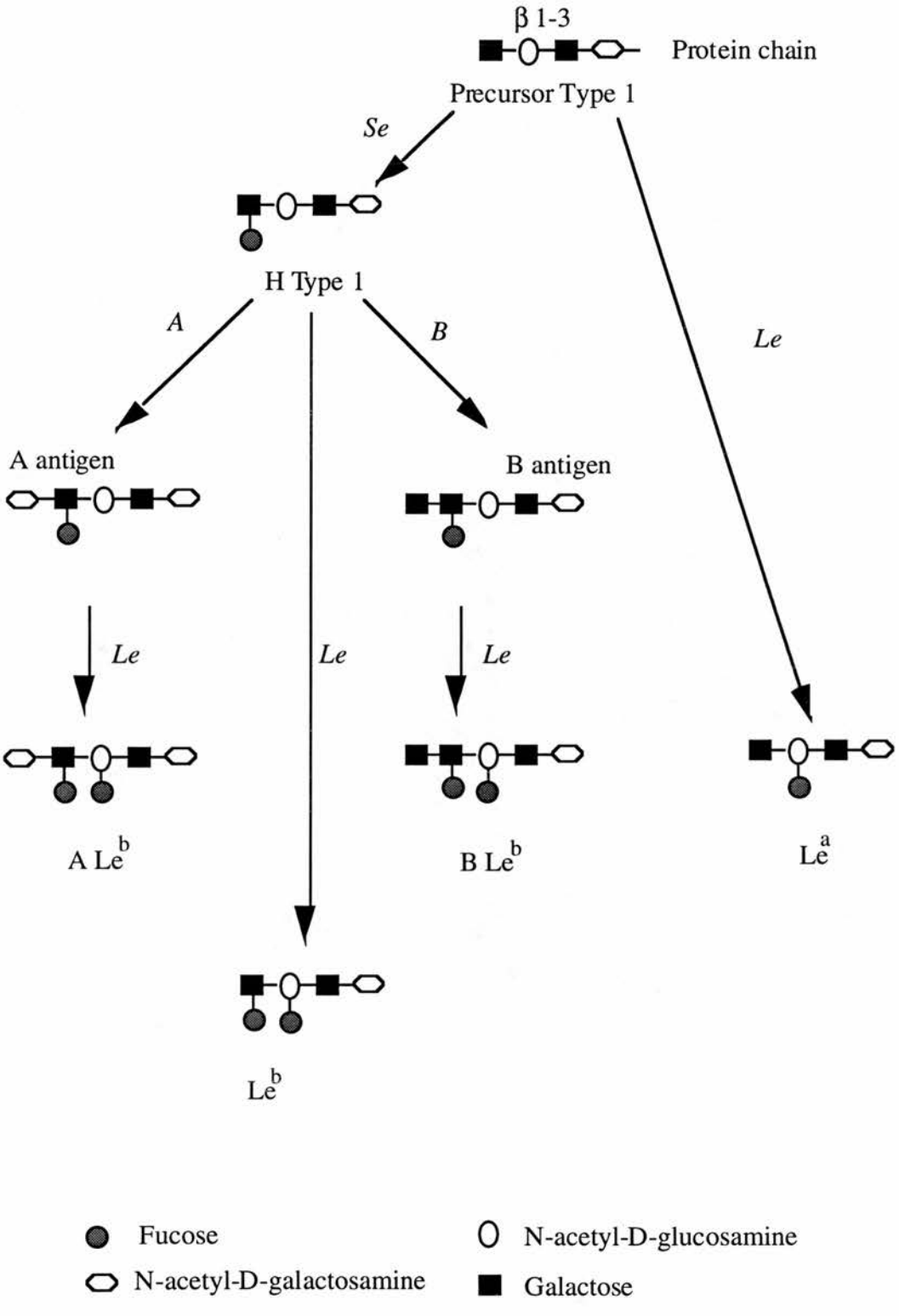


Figure 1.3 Production of H, A, B and Lewis antigens from precursor type 1

1.9.6 Blood group antigens act as receptors for microorganisms

There is evidence that blood group antigens can act as receptors for some microorganisms and this might explain some of the association reported between blood group and disease [Mourant *et al.*, 1978]. Table 1.7 summarises the blood group antigens that act as receptors for microorganisms [Blackwell, 1989]. The Duffy antigen has been demonstrated *in vitro* to be a receptor for *Plasmodium knowlesi* which is closely related to *P. vivax* [Miller *et al.*, 1975]. This antigen is expressed in white populations but is absent in the majority of African and American Blacks [Sanger *et al.*, 1955] and this might explain why African Blacks are more resistant to malaria infection caused by *P. vivax* [Miller *et al.*, 1975]. P blood group was reported to be the receptor for fimbrial adhesins expressed on strains of *E. coli* [Kallenius *et al.*, 1980]. L-Fucose containing glycoconjugates are proposed as epithelial receptor for *Candida albicans* on the basis of adhesion inhibition studies using sugars and lectins as blocking agents [Critchley and Douglas, 1987]. Le^a, H type 1 and H type 2 blood group antigens have been proposed as possible epithelial cell receptors for *C. albicans* [May *et al.*, 1989; Brassart *et al.*, 1991; Tosh and Douglas, 1991; Cameron and Douglas, 1996]. The Le^b blood group antigen has been reported to mediate *H. pylori* attachment to human gastric mucosa. Soluble glycoprotein presenting the Le^b antigens or treatment of cells with antibodies to the Le^b antigens were shown to inhibit bacterial binding [Borén *et al.*, 1993].

Table 1.7 Blood group antigens that act as receptors for microorganisms

Microorganisms	blood group receptors	References
C. albicans	Le^a	May *et al.*, 1989
C. albicans	H type 2	Tosh and Douglas, 1992
H. pylori	Le^b	Borén *et al.*, 1993
E. coli	P	Kallenius *et al.*, 1980
	M	Vaisanen *et al.*, 1982
	N	Jann *et al.*, 1988
	S	Korhonen et al., 1986
H. influenae type B	Anton	Van alphen *et al.*, 1986
S. aureus	Le^a	Saadi *et al.*, 1993
Plasmodium knowlesi	Duffy	Miller *et al.*, 1975

Table reproduced from Blackwell (1989) with modifications

The increased proportion of non-secretors among carriers of some potentially pathogenic bacteria and yeasts might be attributed to the Le^a antigen acting as one of the host cell receptors for some organisms [Blackwell, 1989; May *et al.*, 1989; Rahat, 1990, Tosh and Douglas, 1991; Aly, 1992; Saadi *et al.*, 1993]. Saadi and colleagues [1993] found binding of *S. aureus* to buccal cells correlated with the amount of monoclonal anti-Le^a bound to the cells. Pretreatment of cells with monoclonal anti-Le^a or anti-precursor type 1 antibodies significantly reduced bacterial binding [Saadi *et al.*, 1993]. Alternatively, if the terminal fucose of H type 2 is an important receptor for adhesins of some microorganisms, the terminal fucose of Le^b in mucosal secretions might bind to these adhesins and reduce colonisation.

1.10 Aims of the study and hypothesis to be tested

Blood group O and non-secretion were strongly associated with peptic ulcers. Clarke and colleagues in 1955 and 1956 demonstrated that when duodenal and gastric ulcers were classified separately, the association was more strongly associated with group O individuals and non-secretors [reviewed by Mourant *et al.* 1978]. H type 2, the antigen of blood group O, is found on the cells of all individuals except the very rare Bombay phenotype [reviewed by Race and Sanger, 1975]. The studies of Borén and colleagues indicated that Le^b is the receptor for *H. pylori* on mucosal surfaces. As non-secretors do not express this antigen, this hypothesis could not explain the reported increased susceptibility of non-secretors to peptic ulcers. The work presented in this thesis involved three areas of investigations: 1) a clinical survey to assess genetic and environmental factors associated with isolation of *H. pylori* from patients undergoing gastroscopy; 2) laboratory studies of genetic and environmental factors influencing binding of *H. pylori* to epithelial cells, and 3) the effect of ABO blood group on inflammatory responses.

The objectives of the study were:

1 - to assess blood group and secretor status of local patients with peptic ulcer disease;

2 - to determine if *H. pylori* binds to blood group antigens and if environmental factors such as fasting or smoking affect the binding;

3 - to isolate bacterial adhesins that bind to the blood group antigens by the method used for the *S. aureus* adhesin that binds Le^a;

4 - to determine if there are differences in the immune or inflammatory responses to *H. pylori* associated with ABO blood group or secretor status.

Chapter 2 General Materials and Methods

All chemicals were analytical grade obtained from BDH Chemicals Ltd. UK or Sigma UK.

2.1 Buffers and solutions used for enzyme linked immunosorbent assays (ELISA)

2.1.1 Coating buffer (carbonate-bicarbonate buffer)

Coating buffer consisted of sodium carbonate (15 mM), sodium bicarbonate (35 mM) and sodium azide (3 mM) (pH 9.6).

2.1.2 Phosphate buffered saline (PBS)

PBS contained sodium chloride (15 mM), sodium orthophosphate (3.7 mM) and disodium hydrogen phosphate (9. 6 mM) (pH 7.2).

2.1.3 Washing buffer

Washing buffer consisted of (0.1 % w/v) bovine serum albumin (BSA) and (0.05 % v/v) Tween-20 in PBS (pH 7.2).

2.1.4 Blocking buffer

Blocking buffer was prepared by adding BSA (1 % w/v) to PBS.

2.1.5 Phosphate citrate buffer

Phosphate citrate buffer contained sodium hydrogen phosphate (0.1 M) and citric acid (0.1 M) (pH 5).

2.1.6 Substrate solution

The substrate solution used to detect horseradish peroxidase (HRP) labelled antibodies contained 40 μg O-phenylenediamine in 100 ml of 0.1 M phosphate

citrate buffer (pH 5.0) activated immediately before use by adding 40 µl H_2O_2 (30% v/v).

2.1.7 Stopping solution

The stopping solution contained of 12.5 % (v/v) H_2SO_4.

2.2 Gastric adenocarcinoma cell line (Kato III)

Kato III cells were received in ampoules from the European Collection of Animal Cell Cultures. RPMI medium (1 ml) (500 ml RPMI, 5% foetal calf serum, 1% glutamine and 1% penicillin/streptomycin) was added to the ampoule. The cells were transferred to a universal container, 25 ml of medium was added and the tube centrifuged for 10 min at 300 x *g*. Cells were resuspended in 25 ml RPMI in a growth flask and incubated overnight at 37 ^oC in 5% CO2. The cells were collected by scraping the flask with a cell scraper and washed twice in PBS by centrifugation at 300 x *g* for 10 min. The concentration was adjusted by determination of the total number of cells microscopically with a Neubauer counting chamber.

2.3 Determination of ABO and Lewis blood groups

Blood samples were collected from patients and placed in plain tubes without anti-coagulant. The serum was separated and stored at -20^oC. The ABO blood group of the patients was determined from blood specimens by slide agglutination with monoclonal anti-A and anti-B (Scottish National Blood Transfusion Service). Tube agglutination with anti-Lewis^a (Le^a) and anti-Lewis^b (Le^b) (Scottish National Blood Transfusion Service) was used to assess Lewis group for secretor status. Non-secretors cannot express Le^b, therefore patients whose cells were agglutinated by anti-Le^a only were classed as non-secretors and those agglutinated by anti-Le^b as secretors.

2.4 Purification of monoclonal anti-Le^a and anti-Le^b

Synsorb affinity adsorbent (Chembiochem Ltd., Edmonton, Canada) with the synthetic carbohydrate determinant of Le^a or Le^b covalently linked to the silica matrix was used to purify the anti-Le^a and anti-Le^b monoclonal antibodies. Mouse hybridoma culture supernatant (20 ml) containing anti-Le^a antibody (LM 112/161) or anti-Le^b (LM 112/81) antibody was kindly provided by Dr. R. Fraser, Scottish Antibody Production Unit (SAPU) Glasgow and West Scotland Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire, UK. The supernates were mixed with 1g of the corresponding Synsorb beads overnight at 4°C. The unbound antibodies were removed by centrifugation at 50 x *g* for 10 min and the beads were washed twice with PBS by centrifugation at 50 x *g* for 5 min. The bound material was eluted by adding 5 ml of 2% (v/v) ammonia (BDH, 35%) in saline (pH 11) to the beads for 15 min at room temperature followed by centrifugation at 50 x *g* for 5 min. The supernatant was dialysed against PBS overnight and stored at -20°C. The washed beads were stored in ethanol (70 % v/v) at 4°C.

2.5 Monoclonal and polyclonal antibodies

Monoclonal and polyclonal antibodies, the animal from which they were obtained, isotype and source are listed in Table 2.1

Table 2.1 Monoclonal and polyclonal antibodies

Antibodies	Host	Isotype	Source
Monoclonal			
Anti-A	mouse	IgM	SNBTS
Anti-B	mouse	IgM	SNBTS
Anti-Le^a (red cell typing)	mouse	IgM	SNBTS
Anti-Le^b (red cell typing)	mouse	IgM	SNBTS
Anti-Le^a (inhibition studies)	mouse	IgM	SAPU
Anti-Le^b (inhibition studies)	mouse	IgM	SAPU
Anti-H type 2	mouse	IgM	SAPU
Anti-H type 2	mouse	IgM	Serotec
Polyclonal			
*FITC anti-mouse IgG	goat	IgG	Sigma
+HRP-anti-rabbit	donkey	IgG	SAPU
Anti-human IgA	goat	IgA	Sigma
Anti-human IgG	rabbit	IgG	SAPU
Anti-human IgM	rabbit	IgM	SAPU
+HRP-anti-sheep/goat IgG	donkey	IgG	SAPU

* FITC fluorescein isothiocyanate labelled

+ HRP = horseradish peoxidase labelled

2.6 Bacteria

H. pylori, strains NCTC 11637 and NCTC 11916 were obtained from the Central Public Health Laboratory (Colindale) and 51 isolates were isolated from endoscopic biopsies of patients attending the gastroscopy clinic at the Western General Hospital.

2.6.1 Transport, culture media and storage of bacteria

The specimens for microbiological culture were transported to the laboratory in 0.1 ml isotonic sterile saline and cultured on Campylobacter selective medium containing 7% horse blood, 10 mg L^{-1} vancomycin, 5 mg L^{-1} trimethoprim and 5 mg L^{-1} amphotericin B plus 5 mg L^{-1} cefsulodin or 2500 IU L^{-1} polymyxin B or 10,000 IU L^{-1} colistin. The bacteria were incubated at 37°C in anaerobic jars with a microaerophillic atmosphere provided by gas generating kit (*Campylobacter* system BR60, Oxoid, Unipath Limited, Basingstoke, England) for 3 - 7 days. Bacteria were stored at -70°C in 25% glycerol and 10% mucin.

2.6.2 Identification of *H. pylori*

The bacteria were identified as *H. pylori* by Gram stain, oxidase and catalase production and by rapid positive urease test.

2.6.3 Calculation of total bacterial count

A heavy suspension of the bacteria was prepared from growth on plates in PBS and washed three times by centrifugation at 1000 x g for 20 min. The bacterial pellet was resuspended in 2 ml of PBS. The optical density (OD) of two fold dilutions of the washed suspension at 540 nm was determined by a spectrometer (Pye Unicam) and the total number of bacteria was determined in a counting chamber (Thoma) by light microscopy. A graph of the OD versus total count was prepared for each strain.

2.6.4 Standardisation of bacterial concentration for ELISA

The bacteria were harvested from plates with cotton wool swabs, the cell mass was suspended in PBS and washed three times by centrifugation at 1000 x *g* for 20 min. The bacterial concentration was determined from the OD at 540 nm and adjusted (2.6.3) to 3.2 x 10^8 bacteria ml^{-1} in coating buffer.

2.7 Buffers used for determination of bacterial binding to BECs and Kato III cells

2.7.1 Fluorescein isothiocyanate (FITC) buffer

The buffer contained sodium carbonate (0.05 M) and sodium chloride (0.1 M) (pH 9.2). FITC (0.4 mg/ml) (Sigma) was dissolved in the buffer immediately before use.

2.7.2 1% Buffered paraformaldehyde

Buffered paraformaldehyde (1% v/v) was prepared by adding paraformaldehyde (BDH, 97%) to sodium cacodylate (1% w/v) and sodium chloride (0.75% w/v).

2.8 Bradford reagent for protein estimation

Bradford reagent was prepared from Commasie Blue G250 (0.015 w/v) (Sigma), ethanol (4.7% w/v) and phosphoric acid (8.5% w/v) in distilled water.

Chapter 3

Identification of *H. pylori* in patients with reference to genetic and environmental risk factors for peptic ulcers

3.1 Introduction

Several genetic and environmental risk factors for ulcers were identified before the discovery of the link with *H. pylori*. The relationships between genetic factors and ulcer prognosis have been examined since the discovery by Aird and colleagues that there is an association between blood group O and susceptibility to peptic ulcer. Non-secretors are over-represented among patients with a variety of bacterial or fungal infections [Blackwell, 1989] and were reported to be more prone to duodenal ulcers [Clarke *et al.*, 1956].

The diagnosis of *H. pylori* infections is dependent on isolating the bacteria from gastric biopsies obtained at endoscopy or by microscopic identification of the organism in antral gastric biopsies [Blaser., 1987; Dooley *et al.*, 1989]. Because a significance proportion of strains of *H. pylori* were found to be inhibited by some antibiotics in selective medium [Dent and McNulty, 1988], the sensitivity of local isolates to these agents needs to be considered in this type of study.

Smoking is a major risk factor for gastric and duodenal ulcers [Friedman *et al.*, 1974; Kato *et al.*, 1992], a reduction in the healing rate of gastric and duodenal ulcers [Doll *et al.*, 1958; Korman *et al.*, 1981] and an increase in recurrence of duodenal ulcers [Sontag *et al.*, 1984; Korman *et al.*, 1983]. Since the use of tobacco impairs the immune system [Holt, 1987; Cope and Heatley, 1992] and also enhances colonisation by some bacteria [Fainstein and Musher, 1979], it was considered that current cigarette smokers might be predisposed to *H. pylori* infection. The oral hygiene of smokers is impaired by the residues of tobacco smoke. While it has been reported that *H. pylori* was isolated from dental plaque in gingival crevices or identified by PCR [Krajden *et al.*, 1989; Majmudar *et al.*, 1990; Khandaker *et al.*, 1991], there has been no systematic examination of these sites in relation to smoking.

Patients infected with *H. pylori* develop a systemic antibody response [Pérez-Pérez *et al.*, 1988] and serological assays have been reported to have high sensitivity and specificity compared with endoscopic culture and biopsy [Pérez-Pérez *et al.*, 1988; Talley *et al.*, 1991].

The specific aims of this part of the study were: 1) to compare the sensitivity of local isolates to the 3 selective antibiotics cefsulodin, polymyxin B or colistin; 2) to study prospectively the possibility that dental plaque could harbour *H. pylori*; 3) to compare isolation of the bacteria from biopsies with results of a whole cell ELISA for specific antibodies in serum and saliva; 4) to assess the prevalence of *H. pylori* infection in relation to age, ABO blood group, secretor status and cigarette smoking among patients with dyspeptic symptoms.

3.2 Patients and methods

3.2.1 Patients

Patients attending outpatient endoscopy clinics at the Western General Hospital, Edinburgh, Scotland were randomly selected for the study. Informed consent was obtained and each patient answered a standardised questionnaire to determine age, history of symptoms of dyspepsia, previous and current medication for symptomatic control, use of non-steroid anti-inflammatory drugs and antibiotics in the previous 3 months, history of cigarette smoking and dental history (wearing of dentures, oral hygiene and dental care). The questionnaire (appendix A) was completed by patients prior to endoscopy.

3.2.2 Collection of blood samples

Blood samples were collected from patients as described (2.3). ABO and Lewis blood groups for each patient were determined (2.3). The serum was separated and stored at -20^{o}C for immunoglobulin determination.

3.2.3 Collection of saliva and dental plaque

Saliva (2 - 5 ml) was collected in sterile universal containers prior to endoscopy and dental plaque was removed from gingival sides of the canine and molar teeth of each patient with a sterile dental curette. The specimen was placed in a tube containing 0.1 ml sterile saline solution and plated onto culture medium within 2 h. For patients with full dentures, dental plaque scrapings were taken from false teeth.

3.2.4 Gastric and duodenal biopsies

Two biopsies were collected from the gastric antrum followed by two further biopsies from the body of the stomach and one biopsy from the duodenum. One

biopsy from each site was sent for histology, culture and an additional biopsy was taken from the antrum for the rapid urease test (CLO test). The antral biopsy was taken within 2 cm of the pyloric ring. Samples for culture were placed in the neck of a sterile glass bottle containing 0.1 ml sterile saline to maintain a moist atmosphere but without destroying the mucus layer within which the *H. pylori* are to be found [Dent and McNulty, 1988]. The samples were inoculated onto selective medium containing 7% horse blood, 10 mg L^{-1} vancomycin, 5 mg L^{-1} trimethoprim and 5 mg L^{-1} amphotericin B plus one of the following: 5 mg L^{-1} cefsulodin; 2500 IU L^{-1} polymyxin B; or 10,000 IU L^{-1} colistin (2.6.1). The plates were incubated at 37°C in anaerobic jars with a microaerophilic atmosphere provided by a gas generating kit (*Campylobacter* system BR60) for 3 - 7 days (2.6.1) The bacteria were identified as *H. pylori* by Gram stain, oxidase and catalase reactions and by the rapid positive urease test.

3.2.5 Production of rabbit antiserum to *H. pylori*

A male white New Zealand rabbit was inoculated with a washed suspension of *H. pylori* NCTC 11637. Blood (5 ml) was collected before the first inoculation with 1 ml of 2.5 x 10^8 bacteria ml^{-1} in PBS mixed with complete Freund's adjuvent. The immunisation was repeated after one week with the same concentration of the bacterial suspension in incomplete Freund's adjuvent. After one month, the third injection of the same concentration of bacteria in PBS was given. A week following the last immunisation, blood was collected in a sterile container and serum obtained by centrifugation.

3.2.6 Whole cell ELISA for determination of antibodies to *H. pylori* in immune rabbit serum

H. pylori NCTC 11637 grown for 5 days under the conditions described (2.6.1) were collected and suspended in PBS. The bacteria were heated at 100°C for 60 min

then centrifuged. The pellet was resuspended in coating buffer (2.1.1) and standardised by optical density to a total count of 3.2×10^8 (2.6.3).

Batches of 96 well microtitre plates were coated with 100 µl of *H. pylori* NCTC 11637 in coating buffer and incubated overnight at 4°C. The plates were washed with washing buffer (2.1.3) and blocked with blocking buffer (2.1.4) at room temperature for 20 min. The blocking buffer was removed and the plates washed 6 times with washing buffer.

Non-immune and immune rabbit sera were diluted in blocking buffer and added to the wells in two different plates, one for determination of IgG and the other for determination of IgM and incubated at 37°C for 60 min. The plates were then washed 6 times with washing buffer and goat HRP-anti-rabbit IgG (SAPU) or goat HRP-anti-rabbit IgM (SAPU) diluted 1/200 in blocking buffer were added to the plates and incubated for 60 min at room temperature. After washing 6 times in washing buffer, 100 µl of the substrate (2.1.6) activated immediately before use with 40 ml H_2O_2 (30% v/v) was added. The colour change was stopped after 10 - 20 min by adding 100 µl of H_2SO_4 (2.1.7). The (OD) at 490 nm was determined by an ELISA reader (Dynatech) and corrected by subtracting the OD of the corresponding blank well containing bacteria, HRP-anti-rabbit IgG or HRP-anti-rabbit IgM and substrate.

3.2.7 Whole cell ELISA for detection of antibodies to *H. pylori* in serum and saliva from patients

Batches of 96 well microtitre plates were coated with 100 µl of *H. pylori* NCTC 11637 and incubated overnight at 4°C as described in 3.2.6. The plates were washed with washing buffer and blocked with blocking buffer. Serum samples from patients were diluted 1/100 for IgG, IgM and 1/20 for serum IgA in blocking buffer and patients saliva diluted 1/5 in blocking buffer. These were added to the wells in

different plates for detection of IgA, IgM or IgG and incubated at 37°C for 60 min then washed with washing buffer. Dilutions (1/200) of rabbit HRP-anti-human IgG (Sigma), rabbit HRP-anti-human IgM (SAPU) or rabbit HRP-anti-human IgA (Sigma), (100 µl) were added to separate plates and incubated for 60 min at room temperature. The assay was continued in the same way as the ELISA for determination of rabbit antibodies to *H. pylori*. The assays for all patients were examined at the same time under the same conditions for each of the serum isotypes. All saliva specimens were examined at the same time under the same conditions for salivary antibodies.

3.2.8 Statistical methods

The statistical analysis of the data was performed with Statview SE/Macintosh. The significance levels for differences between two groups were examined with Mann-Whitney U test and Kruskal-Wallis test for three or more groups. Chi Square test was used for prevalence of infection and a P value of 0.05 was regarded as significant.

3.3. Results

3.3.1 Correlation between CLO, culture and specific IgG for identification of *H. pylori*

From one hundred and twenty patients, 11 patients were withdrawn because biopsies were not taken so the results could only be analysed in one hundred and nine patients. Fifty-two patients (47.7%) were found to be *H. pylori* positive on culture and/or the Gram stain from the stomach body and antral biopsies; and among the antral biopsies 48 (92%) were CLO test positive but only 8/52 (15%) duodenal biopsies were positive on culture.

Table 3.1 shown the clinical characteristics and endoscopic finding in patients who were *H. pylori* positive or negative. There were no differences in sex distribution or dental history between the two groups. There was no peptic ulcer disease in *H. pylori* negative patients. Forty patients had peptic ulcer or gastritis; among these 87% were *H. pylori*-positive by culture from stomach body and antrum biopsies, 77% were *H. pylori*-positive identified by CLO and 80% *H. pylori*-positive by specific IgG. Twenty four patients had oesophageal disease, among these 34% were *H. pylori*-positive by culture, 29% were *H. pylori*-positive identified by CLO and 50% *H. pylori*-positive by specific IgG. Forty-five patients had normal endoscopic findings, among whom 20% had *H. pylori* recovered by culture, 17% identified by CLO and 37% had specific IgG. *H. pylori* was not isolated from plaque obtained from the gingival crevices of any of the 109 patients.

3.3.2 Distribution of age among *H. pylori*-positive patients

Age distribution was assessed among *H. pylori*-infected patients. *H. pylori*-positive patients had a mean age of 58 years which tended to be older than *H. pylori*-negative patients, mean age 46 years, The prevalence of *H. pylori* infection increased from

30% in those between the ages of 20 to 30 years to 47% in those between the ages of 40 and 50 years. This increased to 65% at the age of 60 years then decreased to 50% in those between 70 and 80 years. By the Chi square test, these differences were not significant (df = 6, X^2= =5.42, p = 0.49) (figure 3.1)

Table 3.1 Detection of *H. pylori* among patients referred to gastroscopy clinic

	Peptic ulcers n = 40 (%)	**Oesophageal disease n = 24 (%)**	**Normal n = 45 (%)**
H. pylori	**+ve**	**+ve**	**+ve**
CLO	31 (77)	7 (29)	8 (17)
Culture	35 (87)	8 (34)	9 (20)
Specific IgG	32 (80)	12 (50)	17 (37)

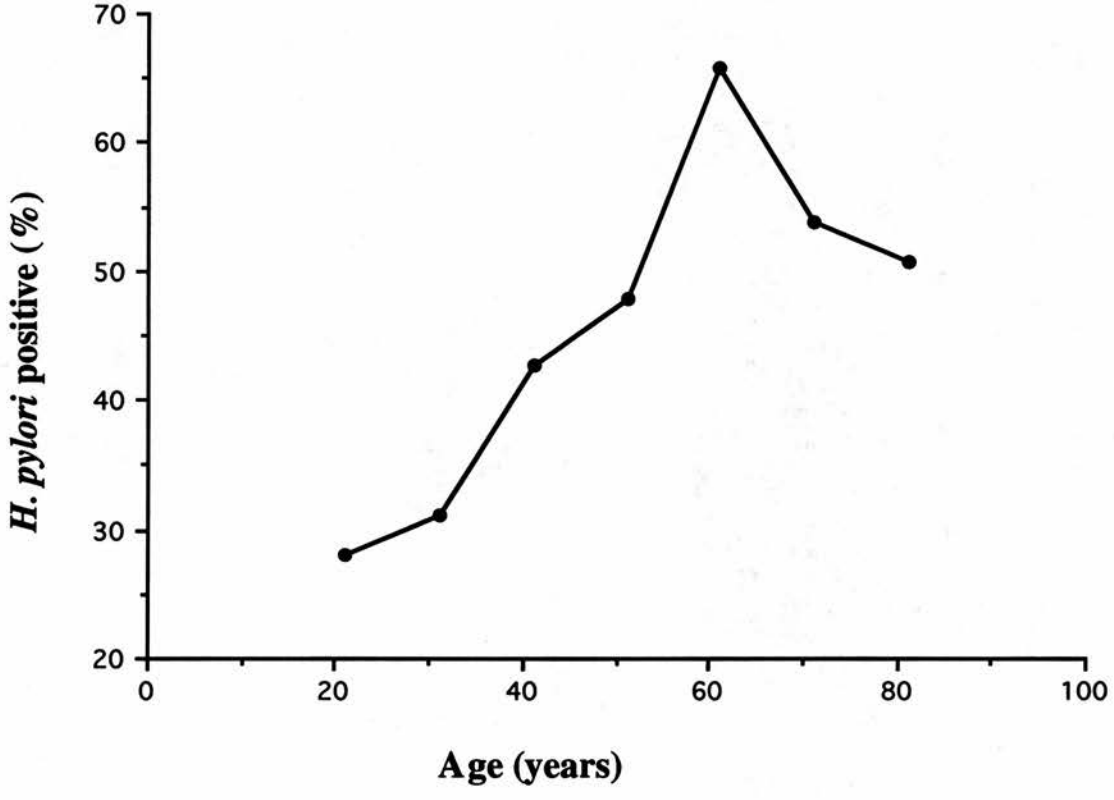


Figure 3.1 Age distribution among patients with *H. pylori* infection

3.3.3 Distribution of blood group and secretor status in relation to *H. pylori* infection

Among 111 patients refered for gastroscopy, 62% were blood group O, 26% were blood group A, 7% blood group B and 5% were blood group AB. The endoscopic diagnosis found antral gastritis, duodenitis, gastric ulcer or duodenal ulcer in 40 (41%) patients. These were considered to have significant gastroduodenal disease but excluded oesophageal disease. Blood group O patients were more likely to have endoscopically visible gastroduodenal disease 69% compared with 31% of other blood groups and blood group O patients were more likely to have duodenal ulcer compared with other disease category, but the differences were not significant (Table 3.2) ($df = 2$, $X^2 = 0.84$, $p = 0.6$).

H. pylori was isolated from 33 (49%) of 68 blood group O and 19 (44%) of the 43 patients of other blood groups, but there was no significant prevalence of *H. pylori* infection among blood group O patients ($df = 1$, $X^2 = 2.57$, $p = 0.1$) (Table 3.3).

Non-secretors had more gastroduodenal disease 19/37 (51%) compared with secretors 20/42 (47%) but this was not significant ($df = 1$, $X^2 = 0.06$, $p = 0.8$) (Table 3.2). The isolation rate of *H. pylori* for the two groups was similar, 26/52 (50%) from secretors and non-secretors.

3.3.4 Distribution of *H. pylori* among smoker and non-smoker patients

Among 109 patients there were 40 patients who were smokers, 38 patients who were non-smokers and 31 patients who were ex-smokers. There were no significant differences observed in relation to prevalence of gastroduodenal disease among smokers. Among 52 *H. pylori* infected patients, there were 18 (34%) patients who smoked, 20 (38%) non-smokers patients and 14 (26%) ex-smokers, but there was no significant difference between the 3 groups (Table 3.4).

A higher prevalence of *H. pylori* infection was associated with the older age groups. Among patients referred for gastroscopy, smokers were younger (mean age = 43 years) compared with non-smokers (mean age = 52 years) and for ex-smokers (mean age = 58 years). There was a higher prevalence of infection among smokers compared with non-smokers matched by age but this was not significant (df = 4, X^2 = 1.47, p = 0.83) (figure 3.2). There were significant increases in prevalence of *H. pylori* infection associated with continuous years of smoking compared with patients who were *H. pylori* negative (df = 4, X^2 = 14.36, p = 0.006) (figure 3.3). There were no significant differences in *H. pylori* infected patients observed for ex-smokers in relation to age (df = 4, X^2 = 3.88, p = 0.42) or years during which they smoked (df = 2, X^2 = 4.1, p = 0.12).

Table 3.2 Distribution of ABO blood groups and secretor status among patients in relation to disease category

	Group O	not- O	Secretors	non-secretors
	n (%)	n (%)	n (%)	n (%)
Duodenal ulcer n = 18	14 (77)	4 (22)	8 (44)	10 (55)
Pyloric ulcer n = 12	6 (50)	6 (50)	6 (50)	6 (50)
Gastritis & Duodenitis n = 10	7 (70)	3 (30)	7 (70)	3 (30)

Table 3.3 Distribution of ABO blood groups among patients in relation to *H. pylori* infection. HP+ = *H. pylori* present by culture and HP- *H. pylori* absent.

	Blood group							
	O		**A**		**B**		**AB**	
	n	**(%)**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**
*** Control (n = 6662)**	3323	(50)	2410	(36)	715	(11)	214	(3)
Study group (n = 109)	68	(62)	28	(25)	7	(7)	6	(6)
HP + (n = 52)	33	(63)	13	(25)	3	(6)	3	(6)
HP - (n = 57)	35	(61)	15	(27)	4	(7)	3	(5)

* distribution of ABO in population of Edinburgh [Kinane *et al.*, 1982].

Table 3.4 Distribution of *H. pylori* infection among patients with reference to smoking status and gastroduodenal disease. HP+ = *H. pylori* present by culture and HP- *H. pylori* absent.

	HP +		**HP -**		**disease**		**Normal**	
	n	**(%)**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**
Number of observations	52	(48)	57	(52)	64	(59)	45	(41)
Current smoker (n = 40)	18	(45)	22	(55)	23	(58)	17	(42)
Ex smoker (n = 31)	14	(45)	17	(55)	20	(65)	11	(35)
Non-smoker (n = 38)	20	(53)	18	(47)	21	(55)	17	(45)

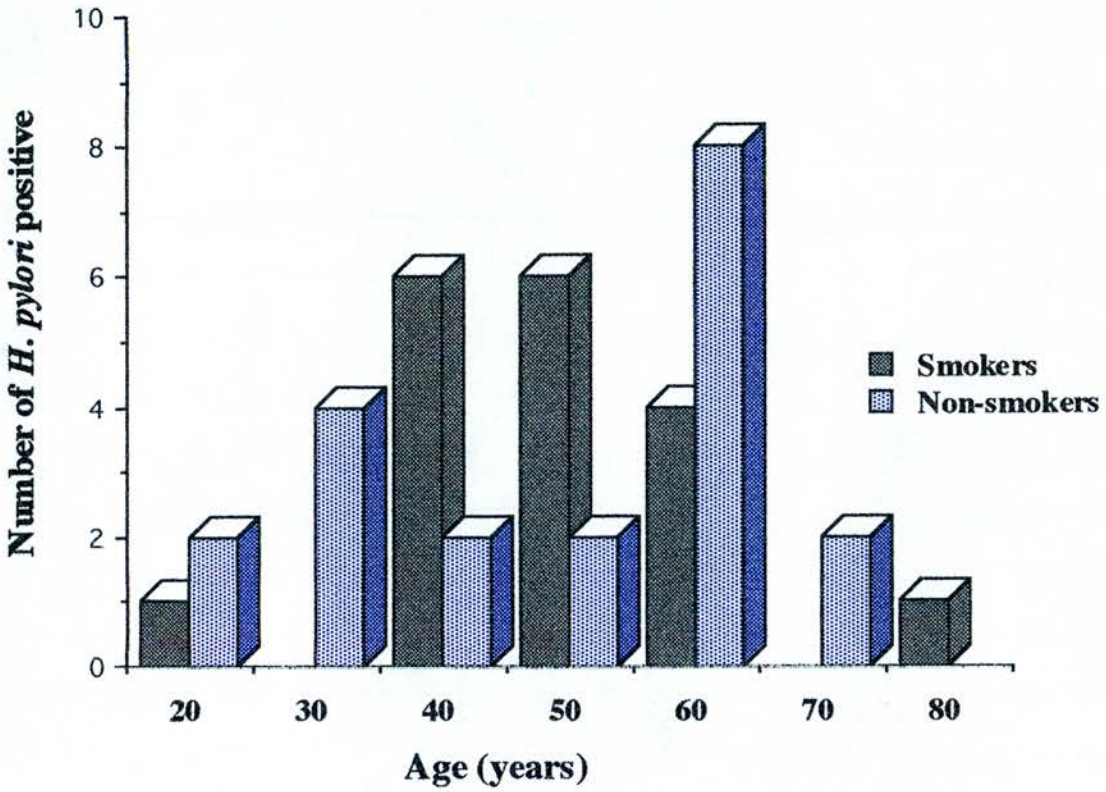


Figure 3.2 Age distribution in relation to *H. pylori* infection among smokers and non-smokers.

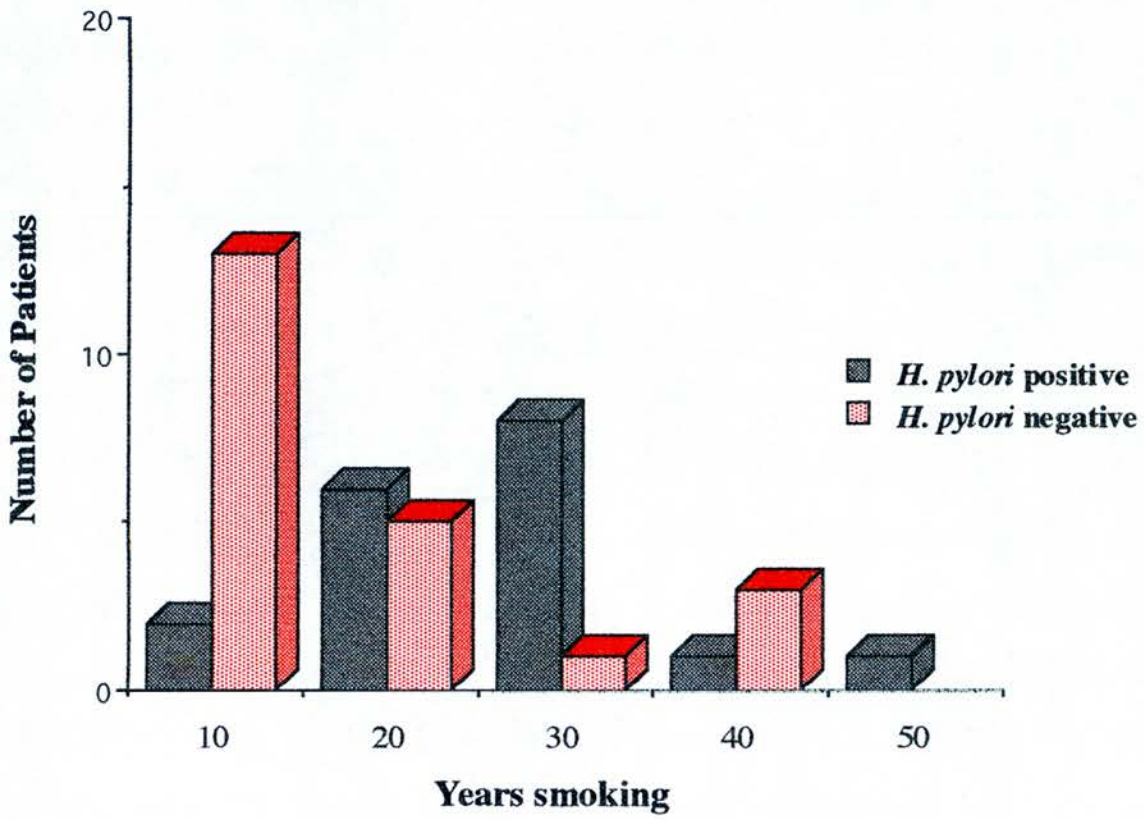


Figure 3.3 *H. pylori* infection among smokers with reference to years of smoking.

3.3.5 Whole cell ELISA for detection of antibodies to *H. pylori* in rabbit serum

Serial dilutions of rabbit serum before and after immunisation with the whole bacteria were prepared and assayed for specific IgG with whole cell ELISA. High absorbency (> 2.050) was observed at dilutions of 1/100, 1/200 and 1/400 of the immune serum but the absorbence of non-immune serum at the same dilution's were 0.185, 0.072 and 0.022 respectively. A series of dilutions of both immune and non-immune serum were used at dilution of 1/800 up to 1/102,400 (figure 3.4).

3.3.6 Determination of serum and salivary antibodies in relation to detection of *H. pylori*

Serial dilutions of serum or saliva were prepared from one patient infected with *H. pylori* and another patient who was negative for *H. pylori* by CLO test, microscopy and culture to optimise the dilution of serum or saliva to be used to determine the levels specific serum IgG, IgM and IgA (figure 3.5, 3.6 and 3.7 respectively) and salivary IgA (Figure 3.8). Dilutions of 1/100 of serum for IgG and IgM or 1/20 for serum IgA and 1/5 of saliva were found suitable for the assays.

All serum samples were screened for specific IgG, IgM and IgA. There were significantly higher OD values of serum IgG and serum IgA levels obtained from patients with *H. pylori* in the antrum identified by culture, microscopy and/or CLO (mean IgG OD 1.060 and serum IgA 0.460) compared with *H. pylori* negative samples (mean IgG OD 0.200 and serum IgA 0.260) ($p < 0.001$ for both). There were no significant differences observed for IgM levels in serum from *H. pylori* infected patients compared with non-infected patients ($p < 0.4$) (figure 3.9).

Saliva specimens were screened at a dilution of 1/5 for specific IgA to *H. pylori* and the results showed significant differences with a mean OD of 0.460 for *H. pylori* infected patients and 0.370 for non-infected patients ($p < 0.01$) (Figure 3.9).

Analysis of the results by the Spearman correlation test found a significant correlation between the levels of serum IgA and salivary IgA ($p < 0.004$).

Patients with peptic ulcers had higher levels of serum IgG (mean absorbance of 0.920) compared with gastritis patients (mean absorbance 0.480), oesophageal diseases (0.450) or no evidence of disease (0.440). By Kruskal-Wallis test, there were significant differences for specific serum IgG ($p<0.001$) but not for serum IgM ($p < 0.7$) , serum IgA ($p < 0.3$ or salivary IgA ($p < 0.2$)(figure 3.10)

Serum and salivary antibodies levels against *H. pylori* was assessed in relation to blood group and secretor status. Blood group A patients had higher level of serum IgG and lower level of salivary IgA compared with blood group O, but this was not significant (by Mann Whitney U test, serum IgG $p< 0.2$ and salivary IgA $p< 0.1$). Patients of group A had significantly higher levels of serum IgA compared with group O ($p < 0.05$). Secretors had lower levels of serum IgG, serum IgA and higher levels of salivary IgA compared with non-secretors, but this was not significant (Mann Whitney U test, IgG $p< 0.2$, serum IgA $p< 0.5$ and salivary IgA $p< 0.2$)(Table 3.5).

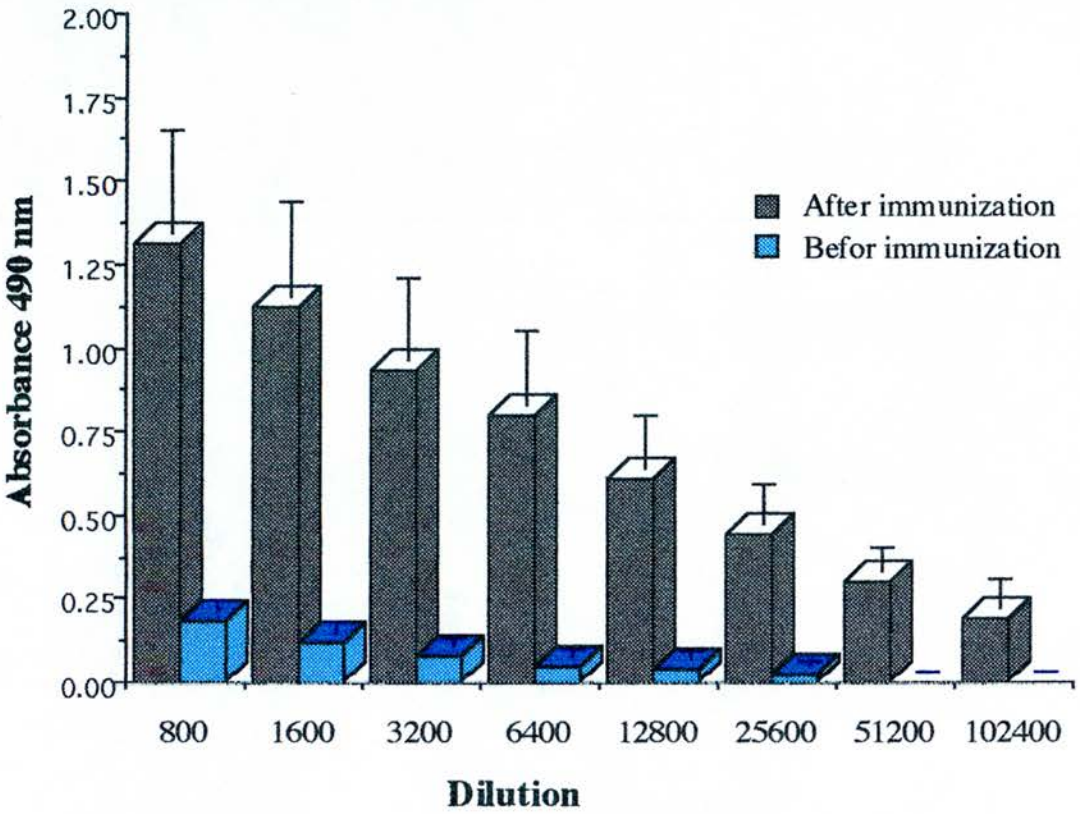


Figure 3.4 Mean of 4 experiments for determination of specific IgG in rabbit serum before and after immunisation.

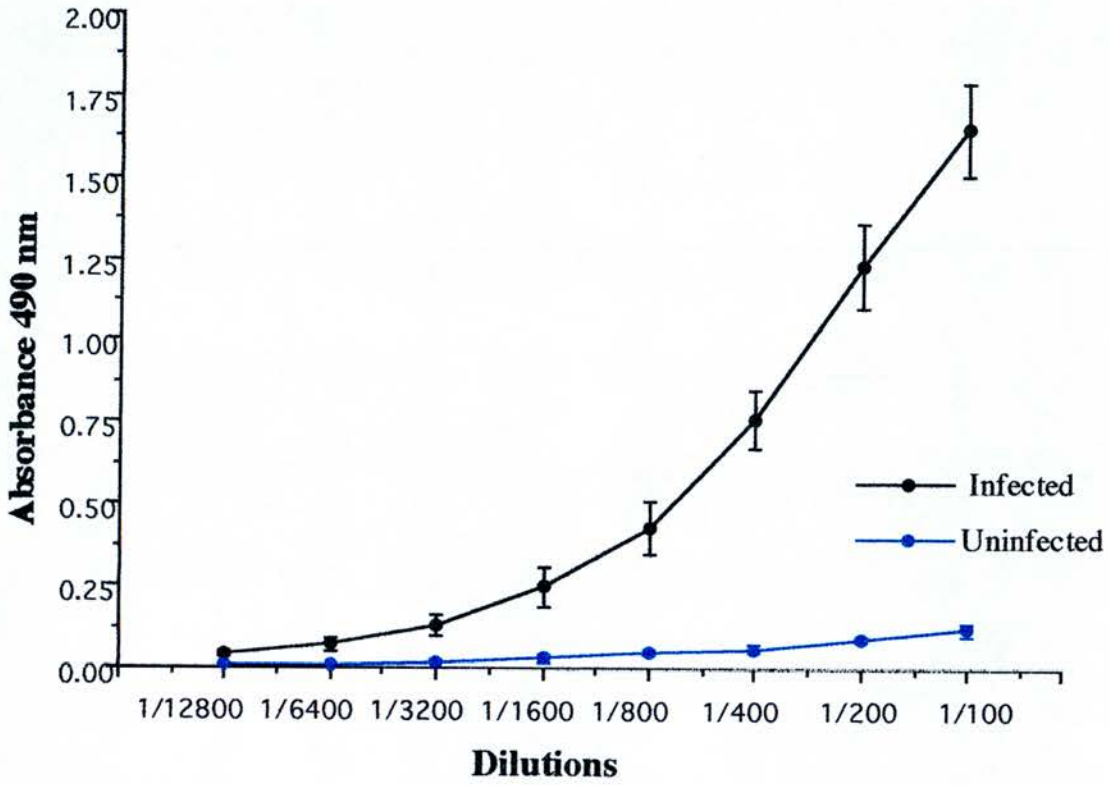


Figure 3.5 Detection of specific IgG to *H. pylori* by whole cell ELISA. Each point represents the mean obtained from three replicates of serum from a patient with *H. pylori* isolated from the antrum and serum from a patient with no evidence of infection.

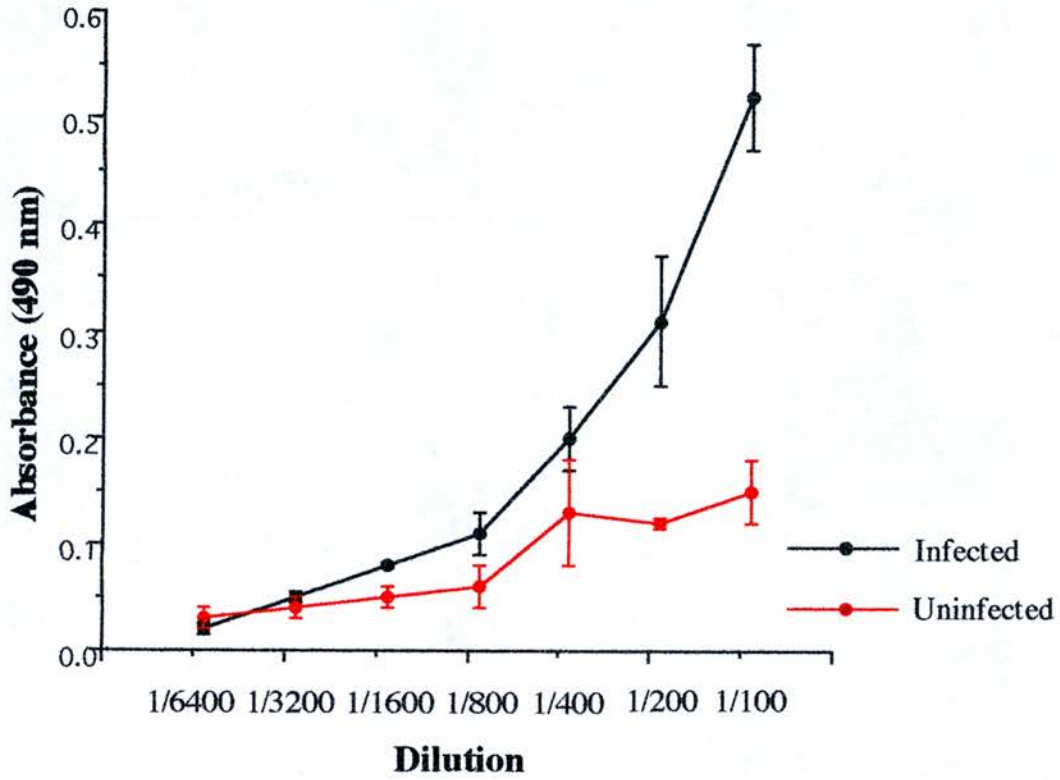


Figure 3.6 Detection of specific IgM to *H. pylori* by whole cell ELISA. Each point represents the mean obtained from three replicates of serum from a patient with *H. pylori* isolated from antrum and serum from a patient with no evidence of infection.

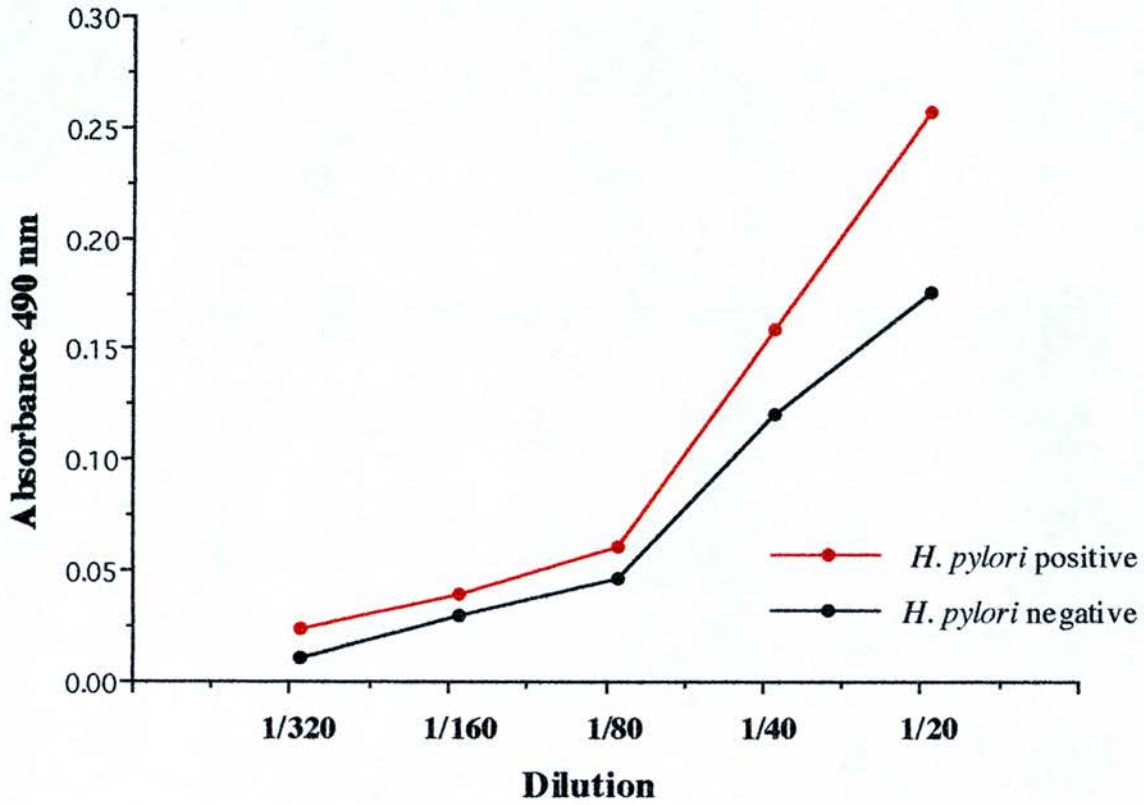


Figure 3.7 Detection of serum IgA to *H. pylori* by whole cell ELISA with serum from a patient with *H. pylori* isolated from antrum and serum from a patient with no evidence of infection.

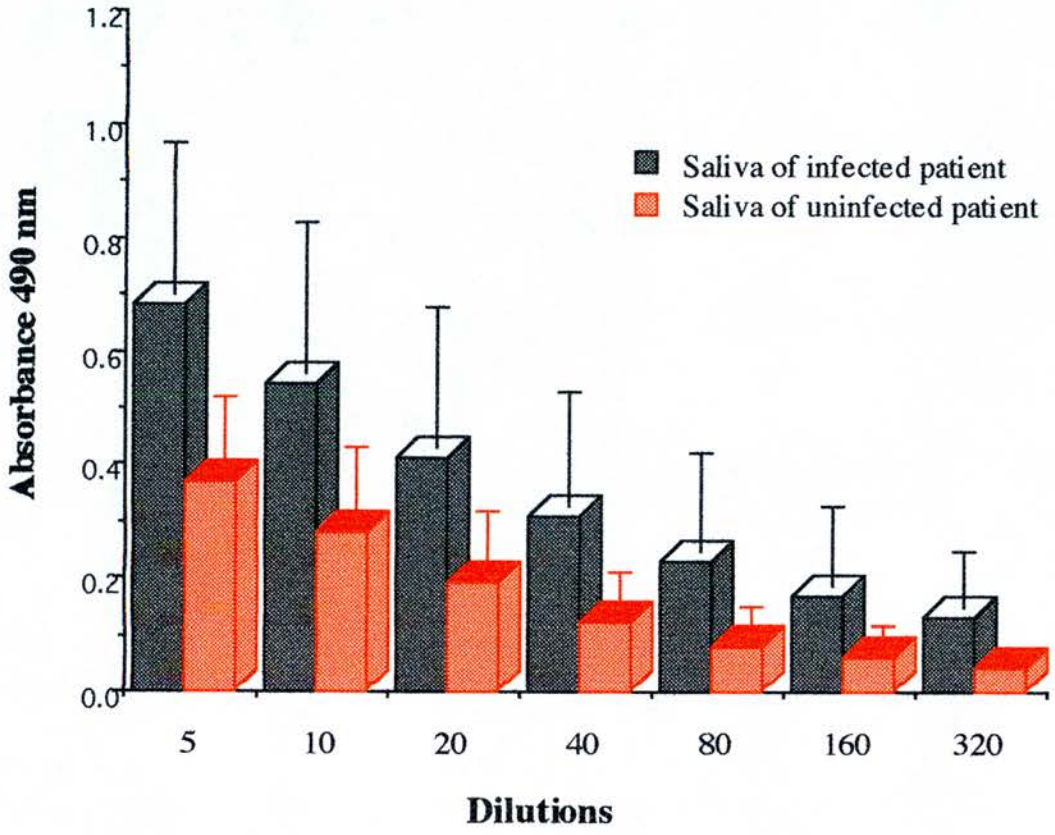


Figure 3.8 Detection of IgA specific for *H. pylori* in saliva by whole cell ELISA. Each point represents the mean obtained from three replicates of saliva from a patient infected with *H. pylori* and saliva from a patient with no evidence of infection.

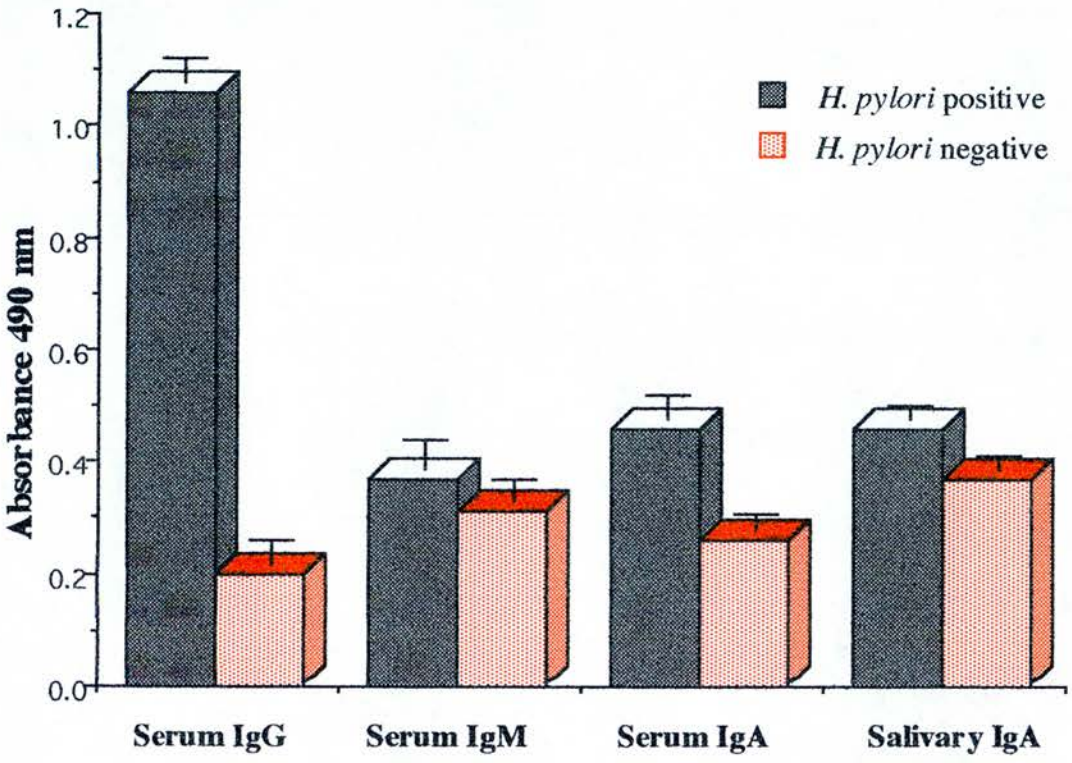


Figure 3.9 Specific serum IgG, IgM, IgA and salivary IgA to *H. pylori* of 109 patients, 52 infected with *H. pylori* (positive) and 57 not infected (negative).

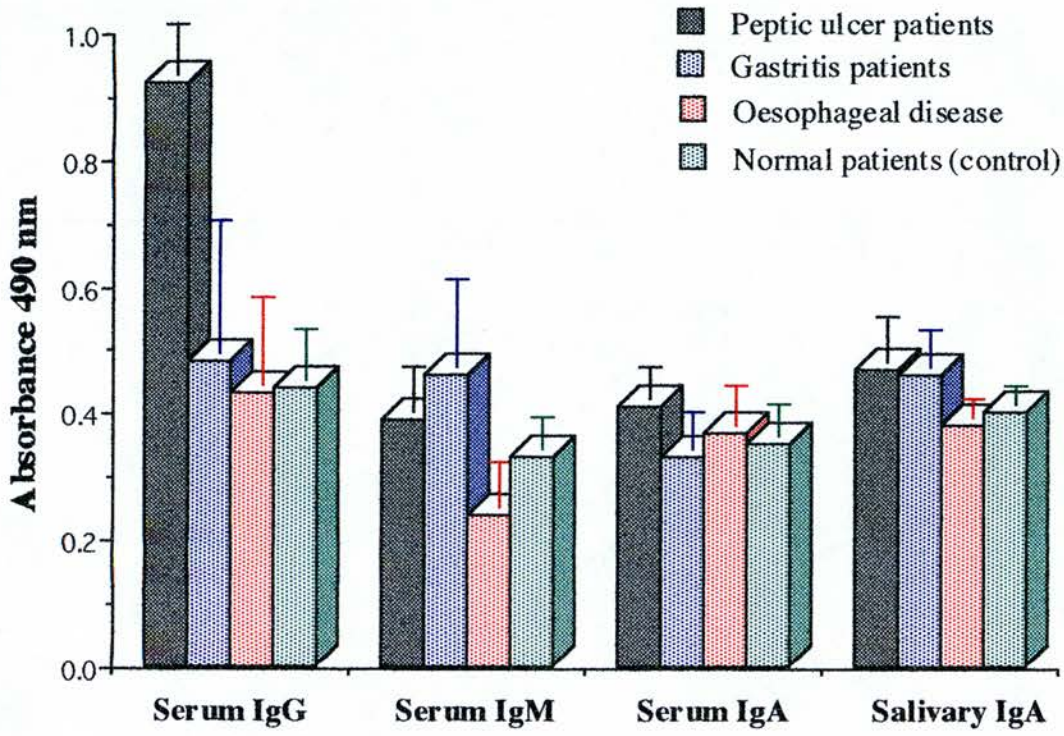


Figure 3.10 Distribution of specific serum IgG, IgM, IgA and salivary IgA to *H. pylori* among patients with different gastroduodenal diseases.

Table 3.5 Mean OD of serum IgG, IgA and salivary IgA antibodies against *H. pylori* in relation to blood group and secretor status among the patients referred to the gastroscopy clinic.

	Group O	**Group A**	**P**	**Secretor**	**Non-secretor**	**P**
Serum IgG	0.610	0.710	0.2	0.560	0.730	0.2
Serum IgA	0.330	0.390	0.05	0.350	0.370	0.5
Salivary IgA	0.440	0.380	0.1	0.430	0.400	0.2

3.4 Discussion

The results of this chapter are assessed in relation to the objectives set in the introduction.

3.4.1 The effect of antibiotics in selective medium

Among 109 patients referred for gastroscopy, there were 52 patients in whom *H. pylori* was identified by CLO test, culture or by stained biopsies. *H. pylori* was identified on plates containing polymyxin B, cefsoludin or colistin. Although many biopsies yield a nearly pure growth of *H. pylori*, a significant proportion of positives may be missed when only non-selective media are used [Goodwin *et al.*, 1985]. Skirrow's medium has been used but is not generally recommended because 14% of the isolates can be inhibited by nalidixic acid, and polymyxin B can inhibit 5% of isolates [Dent and McNulty, 1988]. In the present study only one isolate was inhibited by polymyxin B on primary isolation and subculture, and another strain was inhibited with colistin on primary isolation but it grew on subculture when tested again with colistin. The best results have been achieved with combinations of vancomycin, amphotericin, trimethoprim and colistin or cefsulodin in media supplemented with horse or sheep blood (5% - 10%) or serum with or without charcoal [Dent and McNulty, 1988; Goodwin *et al.*, 1985]. The results of this chapter agree that this combination is optimal for primary isolation of *H. pylori* from gastric biopsies in the local population.

3.4.2 Assessment of dental plaque for culture of *H. pylori*

For an infected individual to transmit *H. pylori*, the organism must leave its protected environment in the gastric mucus layer and pass into the gastrointestinal tract. The evidence for isolation of *H. pylori* from saliva and dental plaque is contradictory [Krajden *et al.*, 1989 and Bernander *et al.*, 1993]. *H. pylori* was

cultured from none of the saliva specimens and from only one dental plaque of 29 patients who yielded positive growth from antral biopsies [Krajden *et al.*, 1989]. In another study, *H. pylori* was isolated from dental plaque of asymptomatic volunteers in India [Majmudar *et al.*, 1990], and Khandaker and colleagues reported the successful isolation of *H. pylori* from gingival crevices and dental plaque of patients with *H. pylori* associated peptic ulcer disease but the specimens were collected after gastroscopy [Khandaker *et al.*, 1991]. Although it had been predicted that *H. pylori* might be isolated from plaque of smokers, the bacteria were not isolated from dental plaque of either smokers or non-smokers collected before gastroscopy. This suggests that the gingival crevices are probably not suitable for growth of these bacteria.

Polymerase chain reaction (PCR) was used for detection of *H. pylori* DNA [Banatvala *et al.*, 1993; Mapstone *et al.*, 1993]. Banatvala and colleagues found *H. pylori* DNA in dental plaque by PCR with a primer for the species specific urease gene. They also detected *H. pylori* DNA in individuals whose antral biopsies were *H. pylori* negative. This could be explained as a false positive result owing to *Helicobacter*- like or other urease producing organisms colonising the oral cavity [Olsson *et al.*, 1993]. The organism has been isolated from gastric juice [Andersen *et al.*, 1988] and a previous study suggested that the oral - oral route of transmission may be possible from infected individuals who have severe gastro-oesophageal disease [Khandaker *et al.*, 1991].

3.4.3 Serum and salivary antibodies levels in relation to identification of *H. pylori*

Antibodies against *H. pylori* have been detected in serum by bacterial agglutination, complement fixation and enzyme-linked immunosorbent assay (ELISA)[Jones *et al.*, 1986; Goodwin *et al.*, 1987]. Among these methods, the ELISA has proved to be

the most sensitive and specific test depending on the nature of the antigen [Evans *et al.*, 1989; Newell., 1987]. Several studies using non-commercial assays have indicated that serology for *H. pylori* antibodies is accurate both as a primary diagnostic procedure and in monitoring the success of treatment of *H. pylori* infection [Pérez-Pérez *et al.*, 1988; Talley *et al.*, 1991]. Studies of *H. pylori* associated gastritis in children by an ELISA or indirect immunofluorescence (IIF) assay found that there were significant decreases in serum IgG and IgA concentrations within 9 - 12 months after successful therapy [Oderda *et al.*, 1989; De Giacomo *et al.*, 1991; Gifone *et al.*, 1993]. The isotypic pattern of *H. pylori* specific antibodies appeared to differ in serum, saliva, gastric and rectal mucosa [Luzza *et al.*, 1995]. *H. pylori* -specific serum IgG titers were higher than serum specific IgA; the opposite was observed in the saliva samples as anti-*H. pylori* IgA titres were higher than specific IgG titres. In the gastric homogenates, specific IgG and IgA were similar [Luzza *et al.*, 1995].

In this study, immunisation of a rabbit with whole bacteria induced specific antibody against *H. pylori.* Specific rabbit IgG was determined by whole cell ELISA. The 1/800 dilution distinguished between *H. pylori* specific IgG and preimmunization levels, the absorbance was very high at dilutions of less than 1/800, therefore 1/800 dilutions of rabbit serum was used in the ELISA assays as positive and negative controls.

H. pylori specific serum IgG, IgM, IgA and salivary IgA in patients were determined by whole cell ELISA. The results confirmed the diagnostic value of ELISA for determination of serum IgG antibodies against *H. pylori* and showed that 1/100 dilutions of serum and 1/5 dilutions of saliva distinguished between *H. pylori* infected and non-infected patients. These findings are in agreement with previous reports [Vaira and Holton., 1989, Kosunen *et al.*, 1992 and Gifone *et al.*, 1993], but different from those reported for adults by using IIF test for specific IgG which

showed overlapping between the *H. pylori* positive and negative groups of patients [Faulde *et al.*, 1991].

A number of ELISA tests using whole bacteria as antigen have been developed for epidemiological and diagnostic purposes [Floegtad *et al.*, 1990]. The assay has proved sensitive and reproducible and has been satisfactory for all bacteria examined. The technique for coating the bacteria is easy to perform and the method can be applied to a wide range of patients for primary diagnostic and epidemiological studies. The results of the ELISA assay correlated well with the presence of *H. pylori* in gastric biopsies and the assay differentiated between patients with and without peptic ulcers. These results suggest that ELISA might be useful for screening patients refered for endoscopy thereby reducing the endoscopy list in the clinic; however, sera samples could also test negative for *H. pylori* antibodies despite a prior infection. It has also been reported that a quarter of gastric cancer patients who tested seronegative in an ELISA, tested positive using Western blotting [Crabtree *et al.*, 1993].

3.4.4 Assessment of risk factors for peptic ulcer disease in relation to clinical and laboratory finding

Prevalence of *H. pylori* infection is associated with increasing age, lower socio-economic status and certain racial groups [Graham *et al.*, 1991]. *H. pylori* infection is more prevalent in the developing world where infection occurs early in childhood and may rise to over 80% in young adults [Sullivan *et al.*, 1990]. In developed countries the prevalence of infection increases with age at the rate of 15% per annum and reaches 60 - 70% by the age of 70 years [Graham *et al.*, 1991]. There are two possible explanations for the correlation between increasing age and infection. First infection may be acquired gradually throughout life; second this could be a cohort

effect reflecting lower socio-economic standards during the childhood period of the elderly population.

This study found that *H. pylori* positive patients tend to be older than non-infected patients and prevalence of the infection increased with increasing age. This is in agreement with data obtained from other epidemiological studies of seroprevalence rates in different age groups in developed countries [Graham *et al.*, 1991; Sitas *et al.*, 1991; The Eurogast study group, 1993]. In this study the peak of *H. pylori* positive patients was found in the group aged between 50 - 60 years old, whereas the rate tended to be lower in older patients. This has been noted previously by other groups [Dwyer *et al.*, 1990; Sandro *et al.*, 1993] and this has been associated with progression of chronic *H. pylori* gastritis to atrophic gastritis in elderly people, because severe gastric atrophy seems to make the stomach inhospitable to *H. pylori* [Goldschmiedt *et al.*, 1991, Kaneko *et al.*, 1992]. There were no significant differences in rate of infection in the older age group among patients with gastroduodenal disease compared with normal histology.

Individuals of blood group O are significantly over represented among patients with gastric or duodenal ulcers compared with patients of the other blood groups for both British and Greek patients with ulcers [Langman and Doll, 1965; Merikas *et al.*, 1986; Mentis *et al.*, 1991]. Mentis and colleagues found no association between presence of *H. pylori* and blood group O [Mentis *et al.*, 1991]. Another study found a correlation between ABO blood group and *H. pylori* infection in 42 patients with rheumatoid arthritis aged 50 - 66 years. Blood group O patients had *H. pylori* in the antral mucosa more frequently than patients of other blood groups [Henriksson *et al.*, 1993]. It is known that most patients with duodenal ulcers are colonised with *H. pylori* [Tytgat and Rauws, 1990] and the association of duodenal ulcers with blood group O is well established [Semble *et al.*, 1987 and Mentis *et al.*, 1991]. Others have claimed that *H. pylori* infection is not associated with the ABO blood group,

but in that study only a serological evaluation of *H. pylori* infection was made [Höök-Nikanne *et al.*, 1990]. In the present study, blood group O was over represented among patients with gastroduodenal disease and among patients with *H. pylori* infection in antral biopsies, but this was not significant. This observation might be due to the small number of patients involved in the study and differences in patient's ages which is a risk factor for *H. pylori* infection.

Non-secretors of blood group antigens was reported to be over-represented among patients with several bacterial or fungal infections or symptomatic carriers (Table 1.6). The increased prevalence of gastroduodenal disease in non-secretors might be associated with an increased susceptibility to *H. pylori* infection. Non-secretion of blood group antigens was a significant risk factor for gastroduodenal disease without ulcers in 101 patients with dyspeptic symptoms studied endoscopically, but was not associated with the presence of *H. pylori* in antral biopsy specimens [Dickey *et al.*, 1993]. Another study in 185 patients with dyspepsia but not peptic ulcer showed no significant association between *H. pylori* infection and secretor status [Chesner *et al.*, 1992]. Similar results were observed among 271 blood donors which found no association between *H. pylori* antibodies and secretor status but endoscopy was not performed [Höök-Nikanne *et al.*, 1990] Mentis and colleagues showed no association between secretor status and *H. pylori* infection or secretor status with duodenal or gastric ulcers among 454 patients assessed by endoscopy [Mentis *et al.*, 1991]. The results of this chapter agree with previous studies; there was not an increase in prevalence of *H. pylori* infection or salivary IgA levels among non-secretors patients compared with secretors. There was a higher level of serum IgG among non-secretors compared with secretors but this was not significant.

Epidemiological studies found that cigarette smoking is associated with an increased risk for developing duodenal ulcer as well as with a greater incidence of ulcer-related complications including death [Trowell., 1934; Doll and Hill., 1964]. Smoking

delays ulcer healing and increases the risk of recurrence [Korman *et al.*, 1981; Ostensen *et al.*, 1985]. The risk of gastric ulcer is increased among current smokers when compared with those who have never smoked, and higher gastric ulcer rates are found among heavy smokers than light smokers [Stemmermann *et al.*, 1989]. Clinical evidence showed that smoking increased the risk of *H. pylori* infection and current cigarette smokers had a higher rate of *H. pylori* infection than non-smokers and ex-smokers [Bateson, 1993]. There was also a strong correlation between active *H. pylori* infection itself and cigarette smoking in the group with normal endoscopy. This has been controversial [Graham *et al.*, 1991; Braverman *et al.*, 1990]. The present study is in agreement with previous findings that smokers are more at risk for gastroduodenal disease, but there was no correlation between *H. pylori* infection isolated from antral biopsies or serum and salivary antibody levels and smoking. Age was found to be an important factor for *H. pylori* infection. The results showed a significant increase of *H. pylori* infected patients among smokers compared by years of smoking, which indicates that smoking and age are a risk factors for *H. pylori* infection.

3.4.5 Conclusion

Most serological studies on *H. pylori* infection have been qualitative rather than quantitative. The present study differed in that a semiquantitative method was used to assess antibody levels. The results reported by Kreuning *et al.*, [1994]. that IgG level correlated with density of colonisation indicate that this work needs to be expanded with larger numbers of patients with different diseases related to *H. pylori* infection.

The risk factors reported for peptic ulceration examined in this study (blood group, secretor status and smoking) did not appear to influence frequency of colonisation by *H. pylori*. The observations by Mentis *et al.*, [1991] and more recently by Atherton

et al., [1996] suggest that density of colonisation is related to development of disease. In the next chapter, flow cytometry was used to address the effects of these risk factors on bacterial binding and density of colonisation of epithelial cells.

Chapter 4

Risk factors for peptic ulcers and binding of *Helicobacter pylori* to human BECs and gastric adenocarcinoma cells (Kato III)

4.1 Introduction

A number of years before the association between *H. pylori* and peptic ulcers was demonstrated, non-secretors and individuals of blood group O were found to be over-represented amongst patients with peptic ulcer [summarised by Mourant *et al.*, 1978]. Studies in this and other laboratories have found that the epithelial cells of non-secretors bind larger numbers of a variety of micro-organisms than cells of secretors : uropathogenic strains of *E. coli* [Lomberg *et al.*, 1986]; *C. albicans* [May *et al.*, 1989]; *S. aureus* [Saadi *et al.*, 1993].

Two blood group antigens common to most individuals have been proposed to act as receptors for micro-organisms, H type 2 and Lewis^a [Blackwell, 1989]. H type 2, the antigen of blood group O, is found on the cells of all individuals except the very rare Bombay phenotype [Race and Sanger, 1975]. The Lewis antigens on epithelial cells are adsorbed from secretions and reflect those present in the body fluids. Individuals who are non-secretors can only express the Le^a antigen whereas secretors predominantly express the Le^b antigen and highly variable amounts of Le^a [Saadi *et al.*, 1993]. Borén and colleagues suggested that Lewis^b is the receptor for *H. pylori* in their experimental system. As the Lewis^b antigen is absent from cells or body fluids of non-secretors, this cannot explain the apparent increased susceptibility of non-secretors to peptic ulcers [Borén *et al.*, 1993]

Since the turn of the century, evidence has been accumulating that peptic ulcers and smoking are strongly associated. There is a significantly increased (about two fold) prevalence of both duodenal and gastric ulcers in smokers compared with non-smokers [Harrison *et al.*, 1979].

The objectives of this part of the study were : 1) to determine if H type 2 and the Lewis blood group antigens were expressed on the Kato III (gastric adenocarcinoma

cell line) and gastric mucosa cells; 2) to test the hypotheses that in addition to Le^b, H type 2 or Le^a were receptors for these bacteria; 3) to determine if secretor status, ABO blood group, smoking or fasting affect the bacterial binding or the expression of blood group antigens.

4.2 Materials and methods

4.2.1 Bacterial strains and culture

H. pylori, strains NCTC 11637 and NCTC 11916 were obtained from the Central Public Health Laboratory (Colindale) and cultured as described in chapter 2 (2.8.1 and 2.9.2).

4.2.2 Labelling of *H. pylori* with FITC

Bacteria were labelled with FITC by a modification of the method of Wright and Jong [1986]. A heavy suspension of bacteria was prepared and washed twice by centrifugation at 1000 x *g* for 20 min. The bacterial pellet was resuspended in 4 ml of freshly prepared FITC (2.6.1). The mixture was incubated at 37°C for 30 min and washed three times with PBS. The pellet was resuspended in PBS and the concentration determined by OD at 540 nm (2.6.3)

4.2.3 Gastric adenocarcinoma cells (Kato III)

Kato III cells were prepared as described in (2.2), washed twice with PBS and the concentration adjusted to 2.5 x 10^5 cells ml^{-1} following determination of the total number of cells microscopically with a Neubauer haemocytometer counting chamber

4.2.4 Buccal epithelial cells (BEC)

Buccal epithelial cells (BEC) were obtained from healthy members of the Medical Microbiology Department or patients from the endoscopy clinic whose ABO blood group and secretor status had been determined. To test the effect of fasting, BEC from healthy male volunteers were collected during Ramadan when they were fasting 12 - 18 h/day and tested in parallel with cells collected from the same individuals when they were not fasting.

The cells were collected by rubbing the inside of the cheeks with cotton swabs. To remove the cells, the swabs were agitated in 10 ml of PBS. The cells were then washed twice in PBS by centrifugation at 300 x *g* for 10 min. The cells were counted in a haemocytometer then adjusted to 2.5 x 10^5 cells ml^{-1} .

4.2.5 Gastric mucosa cells

Gastric mucosa cells were obtained from patients by means of a cytology brush. The gastric cells were washed twice by centrifugation in PBS at 300 x *g* for 10 min and adjusted with a Neubauer haemocytometer to 2.5 x 10^5 cells ml^{-1}.

4.2.6 Binding assay

4.2.6.1 Binding assay by light microscopy

Binding of *H. pylori* NCTC 11637 to BEC was assessed as described by [Mahajan and Panhotra, 1989]. Equal volumes of a BEC (500 µl) and bacterial suspension were mixed together in a plastic tube and the mixture was incubated at 37°C in an orbital incubator for 60 min. The cells were washed three times with PBS by centrifugation at 300 x *g* for 10 min to remove unattached bacteria. Smears were prepared from the centrifuged deposit, air dried, fixed with methanol for 5 min, stained with Gram's stain and examined by light microscopy with an oil immersion lens.

4.2.6.2 Binding assay by flow cytometry

Buccal cells or Kato III cells (200 µl, 2.5 x 10^5) were mixed with 200 µl of dilutions of the FITC-labelled bacteria to produce different ratios of bacteria per cell. To determine the optimal incubation period, the samples were incubated at 37°C for 0, 30 and 60 min with gentle shaking (100 rpm) in an orbital incubator (Gallenkamp). The cells were washed twice with PBS by centrifugation at 300 x *g* for 10 min to

remove unattached bacteria, resuspended in 150 µl of PBS and fixed with 100 µl of 1% buffered paraformaldehyde. They were stored in the dark at 4°C until analysed.

4.2.7 Analysis of cells by flow cytometery

Analysis was done on an EPICS "XL" flow cytometer (Coulter Electronics, Luton, UK) equipped with a 55 W laser using a power output of 200 Mw at 488 nm. The cells were selected from a display of forward angle light scatter versus 90° light scatter by means of a bit map. A minimum of 1000 cells were analysed from each sample. The percentage of cells showing fluorescence greater than the background (cells without bacteria) level was recorded on a one parameter histogram measuring fluorescence on a logarithmic scale. The mean fluorescence channel values for the positive cells were obtained from a conversion table of log fluorescence supplied by the manufacturer (Coulter). The results were analysed by the immunoanalysis program (Coulter), a computer programme that subtracts the values of the control population from the test population at each channel of the two histograms (fig 4.1). The binding index of each sample was calculated by multiplying the percentage of fluorescent cells by the mean channel value (Saadi *et al.*, 1993).

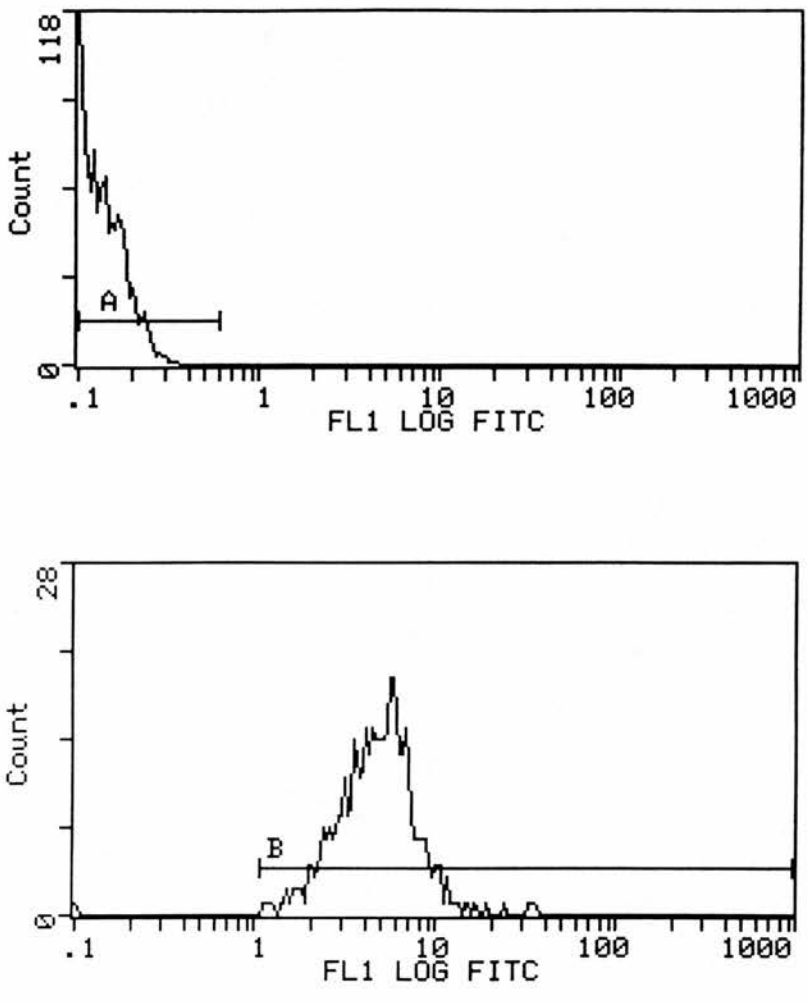


Figure 4.1 Flow cytometric analysis with FITC-conjugated bacteria attached (B) compared to control cells with no bacteria (A)

4.2.8 Detection of Le^a, Le^b and H type 2 on BECs and Kato III cells

Cells were washed and the concentration adjusted to 2.5 x 10^5 ml^{-1}. Cells (200µl) were added to Falcon tubes containing either 200 µl of PBS as control or 200 µl or monoclonal antibodies diluted in PBS: anti-Le^a (1 in 5); anti-Le^b (1 in 5); or anti-H type 2 (1 in 5) (see table 2.1). The tubes were incubated at room temperature for 60 min, the cells were washed twice in PBS at 300 x *g* for 10 min. FITC-conjugated anti-mouse IgM (200µl, 1:200 in PBS) was added to each sample and incubated at 37°C for 30 min with gentle shaking. After washing twice in PBS at 300 x *g* for 10 min, the samples were fixed with 200 µl of 1% buffered paraformaldehyde and stored in the dark at 4°C until analysed with the EPICS-XL flow cytometer.

4.2.9 Inhibition of binding by pretreatment of BEC or Kato III cells with antibodies to cell surface antigens

BEC or Kato III cells were washed and the concentration adjusted as before. The cells (200 µl) were incubated with 200 µl of PBS or dilutions of the monoclonal antibodies against H type 2, Le^a or Le^b in 4.2.8 for 30 min at 37°C with gentle shaking (100 rpm) in an orbital incubator (Gallenkamp) and washed twice to remove unbound antibodies. FITC-labelled bacteria at a concentration of 300 bacteria per cell were added to Kato III cells or BEC pre-treated with PBS or monoclonal antibodies. The cells were washed twice with PBS by centrifugation at 300 x *g* for 10 min to remove unattached bacteria. The samples were resuspended, fixed and stored as before until analysed. Calculation of inhibition of bacterial binding was calculated from the binding indices of test and control sample with the following formula: % of inhibition = 100 - [(test / control) x 100].

4.2.10 Statistical methods

The statistical analysis of the data was performed with Statview SE/Macintosh. The significance levels for differences between two groups were examined with Mann-Whitney U test or by the Rank Sum test.

4.3 Results

4.3.1 Correlation of total bacterial count with optical density

The correlation between the readings of optical density (OD) at 540 nm with total bacteria count for each strain of *H. pylori* tested is shown in figure 4.2

4.3.2 The effect of FITC on bacterial binding

To examine the effect of FITC on binding of the bacteria to BEC, binding of FITC-labelled and unlabelled *H. pylori* NCTC 11637 were examined on 5 different slides by light microscopy; 20 cells were counted for each slide. There were no significant differences in binding of FITC-labelled bacteria (mean 58) compared with unlabelled bacteria (mean 52) at concentration of 100 bacteria per cell.

4.3.3 Optimal conditions for binding and inhibition studies.

Different concentrations of FITC-labelled bacteria were incubated with BEC to determine optimal incubation time, pH and ratios of bacteria per cell. No fluorescence above background levels was observed when bacteria were added to BEC immediately prior to analysis. Attachment increased for the first 30 min then the increase was less rapid as shown in figure 4.3 (mean of 2 experiments); 60 min was used as the standard incubation time for the binding assays.

Attachment of *H. pylori* to BECs was examined with five different concentrations of bacteria / cell (1200, 600, 300, 150 and 75). Figure 4.4 represents the mean of 4 experiments. For further experiments, ratios between 150 - 600 bacteria / cell were used.

Figure 4.5 illustrates the mean of 2 experiments to assess the effect of pH on binding of *H. pylori* to BECs. There were no significant differences between pH 3.9, 6.0 or 7.3; therefore pH 7.3 was used for further attachment assays.

4.3.3 Expression of H type 2 and Lewis blood group antigens on epithelial cells

Binding of monoclonal antibodies to H type 2, Lewis^a and Lewis^b antigens was demonstrated for the Kato III cells, BEC and gastric mucosa cells. Figure 4.6 represents the mean of four experiments to assess binding of monoclonal antibodies against H type 2 and the Lewis antigens to Kato III cells.

Table 4.1 illustrates binding of these antibodies to gastric mucosal cells obtained from four patients. The small number of cells available did not allow repeat studies from the same patient.

Buccal epithelial cells from healthy individuals bound all three monoclonal antibodies and those from individuals of group O (n = 7) had significantly higher binding indices for anti-H type 2 (mean = 1536) when compared by the Rank Sum test with binding indices for non-O donors (n = 7) (mean = 347) ($p< 0.05$) (Figure 4.7). There were no significant differences in binding indices for anti-Lewis antibodies for secretors compared with non-secretors. There were no differences in binding indices for males compared with females.

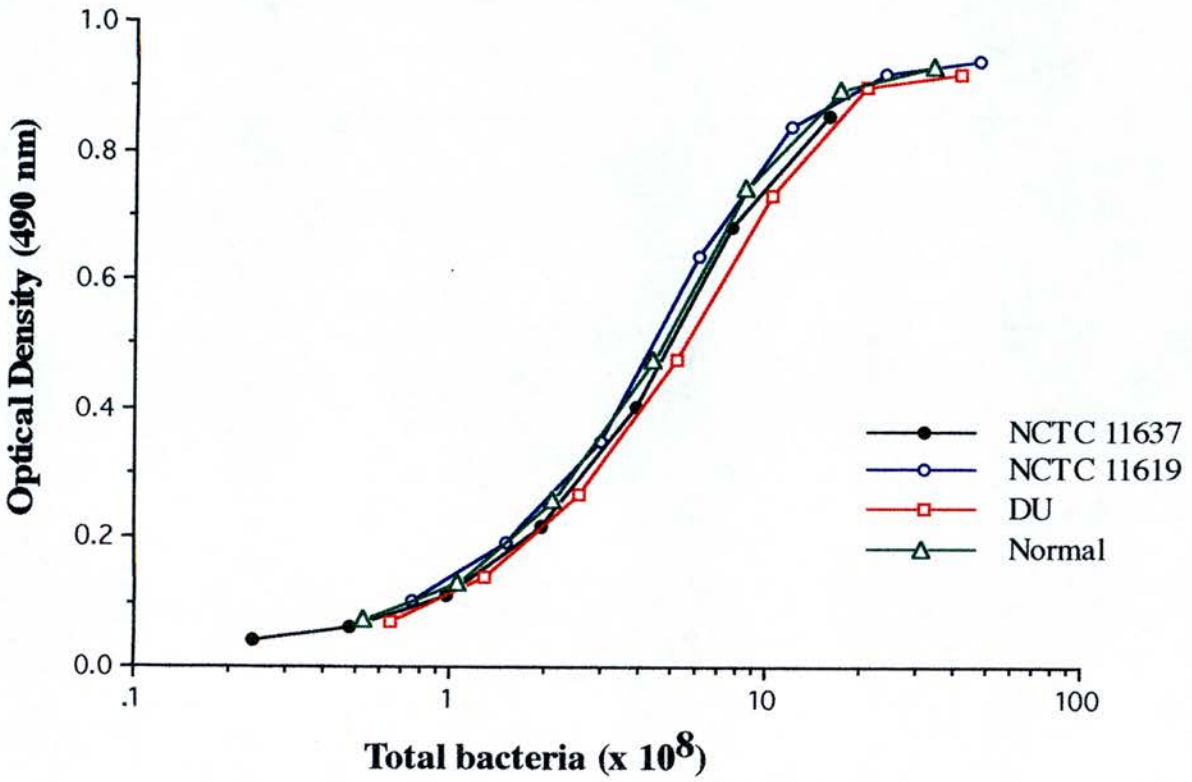


Figure 4.2 Total bacterial count (x 10^8) determined by microscopy versus optical density of *H. pylori* strains NCTC 11637, NCTC 11619, isolates from duodenal ulcer patients (DU) and isolates from normal patients.

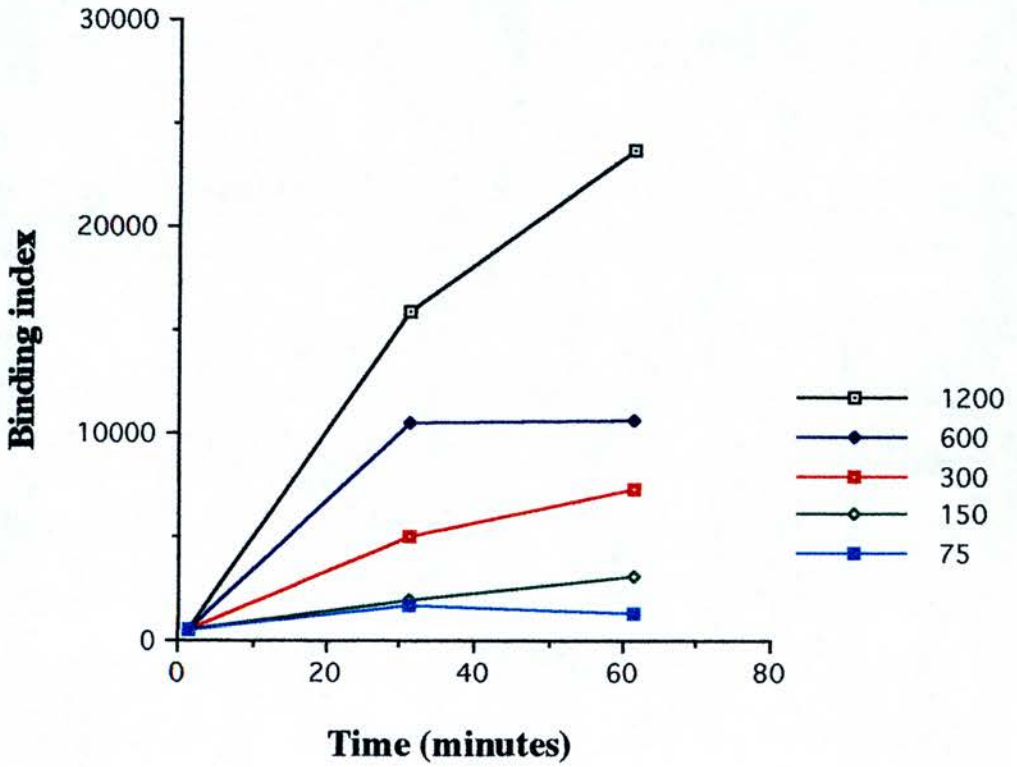


Figure 4.3 Time response of binding of *H. pylori* NCTC 11637 to BEC at the following ratios of 1200, 600, 300, 150, 75 bacteria : cell.

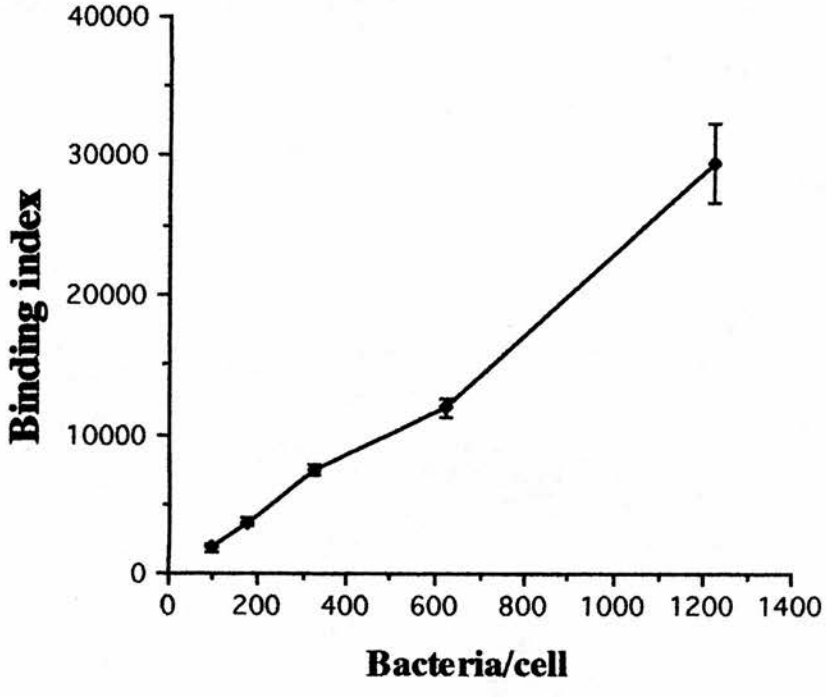


Figure 4.4 Binding index for FITC labelled *H. pylori* NCTC 11637 bound to BEC

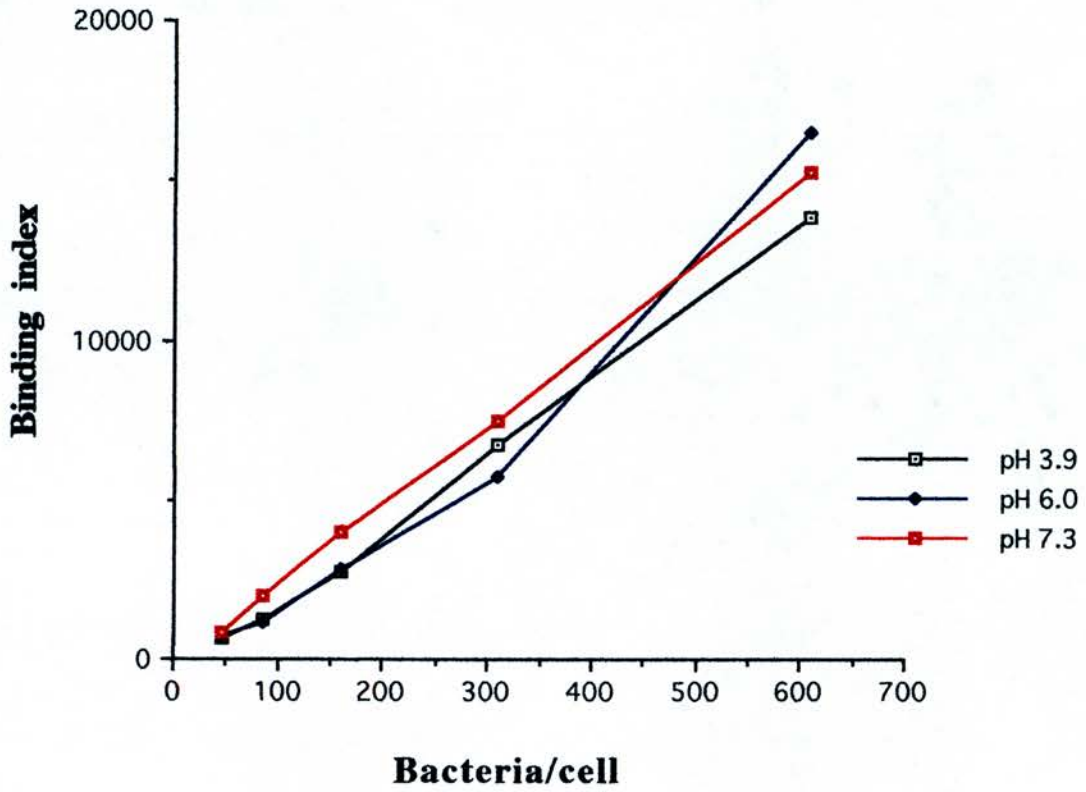


Figure 4.5 pH effect of binding of *H. pylori* NCTC 11637 to BEC

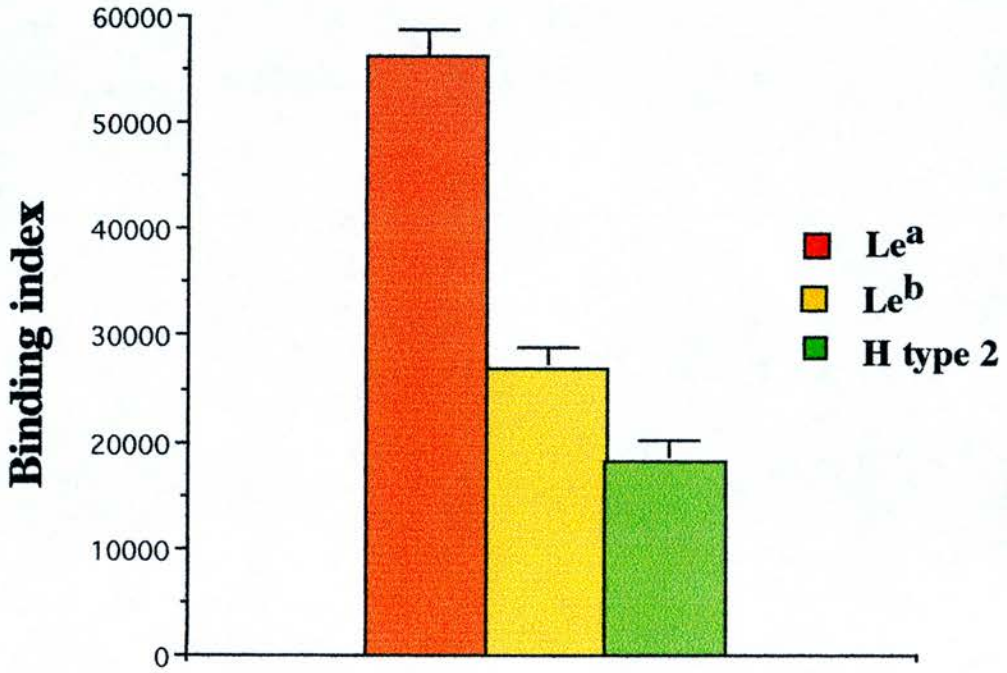


Figure 4.6 Mean binding indices of four experiments to assess the attachment of monoclonal antibodies against Le^a, Le^b and H type 2 to Kato III cells.

Table 4.1. Binding of monoclonal antibodies against Le^a, Le^b or H type 2 to gastric mucosa cells obtained from patients undergoing gastroscopy.

	Le^a		Le^b		H type 2	
Donors	**%**	**mean**	**%**	**mean**	**%**	**mean**
1	94.7	748	89.4	448	59.9	399
2	71.6	686	82.4	604	81.7	603
3	84.1	600	89.9	585	91.1	773
4	81.2	617	90.3	679	82.4	654

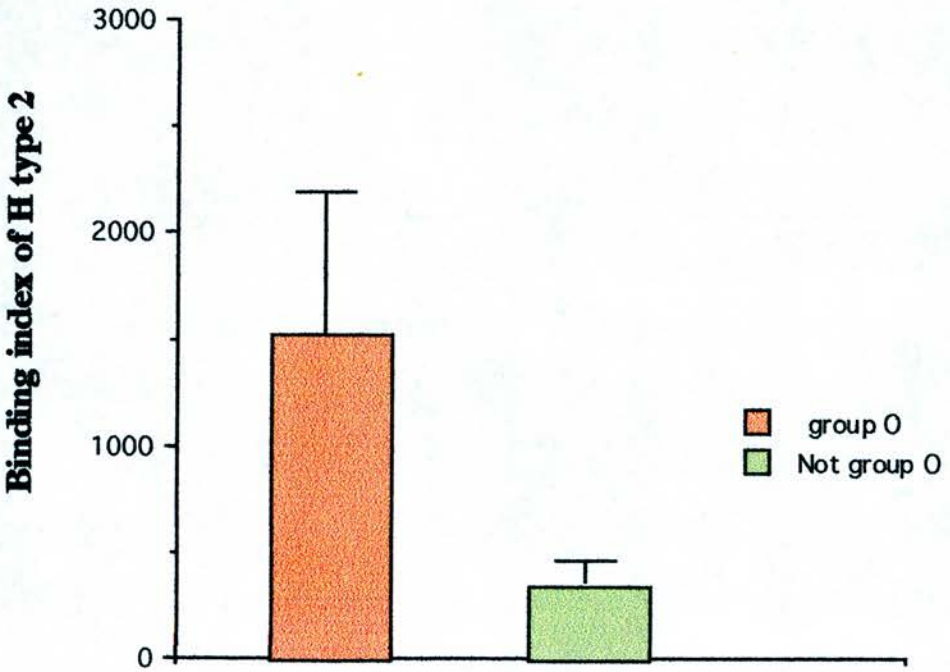


Figure 4.7 Binding of monoclonal antibody against H type 2 to BEC obtained from blood group O (n = 7) and non-O individuals (n = 7)

4.3.4 Binding of *H. pylori* to BEC with reference to ABO blood group and secretor status

Binding experiments with *H. pylori* NCTC 11916 and BEC from 8 secretor and 8 non-secretor donors with no history of gastrointestinal disease found that the mean binding index for secretors was 8669 and for non-secretors was 7118; the mean binding index of *H. pylori* NCTC 11637 to cells of secretors (7925) was also greater than to the cells of non-secretors (5640). By the Rank Sum test, there was no significant different in binding of *H. pylori* to cells from secretors compared with cells from non-secretors. There was no significant difference in detection of the Lewis antigens on BEC of secretors and non-secretors assessed by binding of monoclonal antibodies to the antigens in the flow cytometry assays (Table 4.2). There was a significant difference in detection of H type 2. As the donors had not been matched for ABO groups, the experiment were repeated to assess the effect of ABO blood group.

The experiments were repeated with cells from gastroscopy patients who were non-smokers. The binding indices of *H. pylori* NCTC 11637 with buccal cells from 7 group O secretor patients was significantly greater than with BEC from 7 patients who were non-O secretors ($p<0.05$) (figure 4.8). Binding of *H. pylori* NCTC 11637 to BEC assessed by secretor status found that the mean value for binding indices with cells from 7 group O secretors was significantly greater then the cells from 7 group O non-secretors $p < 0.01$ (figure 4.9).

Table 4.2. Mean binding index of *H. pylori* NCTC 11916 and NCTC 11637 to BECs of healthy donors and expression of blood group antigens (n = number of donors, NS = not significant).

	Secretors n = 8	**Non-secretors n = 8**	**P**
H. pylori NCTC 11916	8669	7118	NS
H. pylori NCTC 11637	7925	5640	NS
Expression Le^a	17406	17754	NS
Expression Le^b	6701	10692	NS
Expression H type 2	1356	334	0.05

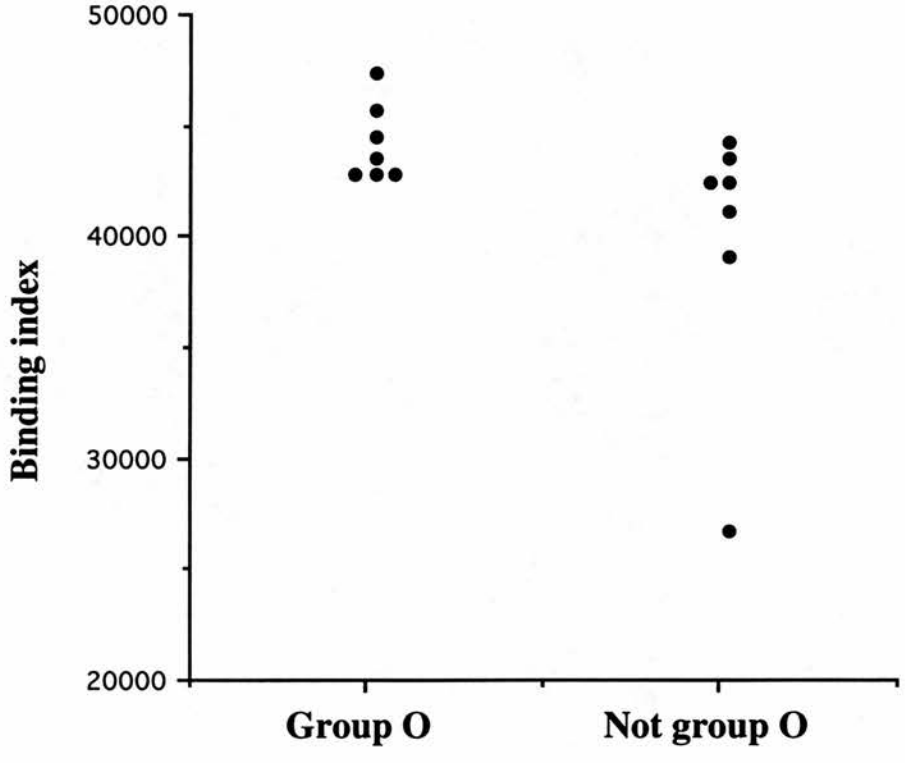


Figure 4.8 Binding of *H. pylori* NCTC 11637 to BEC from patients of blood group O (n = 7) and not group O (n = 7) ($P<0.05$).

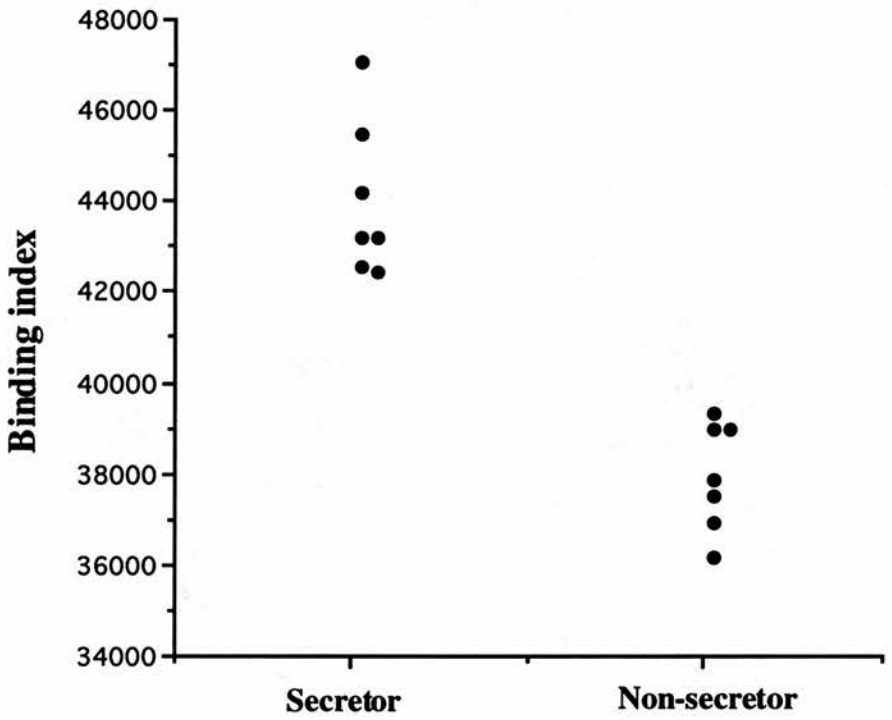


Figure 4.9 Binding of *H. pylori* NCTC 11637 to BEC from patients of blood group O secretors (n = 7) and group O non-secretors (n = 7) ($P<0.01$).

4.3.5 The effect of exposure to cigarette smoke on adherence of *H. pylori* to epithelial cells

Binding of 2 strains of *H. pylori* NCTC 11637 and NCTC 11916 was examined with BEC from students and staff in the Department of Medical Microbiology, 8 were smokers and 8 were non-smokers. The mean binding index of NCTC 11637 for smokers (2392) was higher than that for non-smokers (1510). A similar pattern was found for NCTC 11916; the binding index for smokers (7021) was higher than for non-smokers (5899). By the Rank Sum test, there was no significant difference in binding of *H. pylori* to cells from smokers compared with cells from non-smokers (Fig 4.10)

In another set of experiments with BEC from patients, binding of *H. pylori* NCTC 11637 to cells from 6 smokers and 6 non-smokers was examined. The results showed that the mean binding index for smokers was 68 744 and mean binding index for non-smokers was 58 949; by the Rank Sum test, there was a significant difference in binding of *H. pylori* to BEC cells from smokers compared with cells from non-smokers ($p<0.05$) (Fig 4.11). In another set of experiments, the mean binding index of *H. pylori* to BEC from 7 patients who were smokers (40 301) was significantly higher than to cells of 7 non-smokers (28758) ($p< 0.001$). The smokers had a significantly higher binding indices for anti-H type 2 than non-smokers ($p< 0.02$) but with anti-Lewis^a and Lewis^b the differences were not significant (Table 4.3). There was no significant differences in medication for the patient groups; however, the patients had fasted overnight before the cells were collected; therefore, a larger group of healthy individuals were tested for the effect of smoking and fasting (4.3.6).

4.3.6 The effect of fasting on the binding of *H. pylori* to BECs

As the gastroscopy patients had fasted for approximately 12 hours before collection of cells, the binding studies were repeated with BEC from 30 Muslim males during Ramadan when they were fasting approximately 12 - 16 hr per day. The same donors were examined after the fast when they were eating and drinking normally. Figure 4.12 illustrates the results of 15 smokers and 15 non-smokers for bacterial binding and expression of blood group antigens. By the Rank Sum test there was significantly higher binding of *H. pylori* to cells from non-smokers compared with cells from smokers ($p<0.001$), and there were significantly higher binding indices for monoclonal antibodies to Le^a ($p<0.002$), Le^b ($p<0.005$) and H type 2 ($p<0.001$) on the cell surfaces of non-smokers. For the 30 healthy donors, binding indices for *H. pylori* were significantly correlated with binding indices for anti-H type 2 ($P< 0.005$) and anti-Le^b ($P < 0.001$) but not with anti-Le^a.

Bacterial binding to cells from 7 fasting non-smokers was significantly higher than for cells from 7 fasting smokers ($P< 0.002$) and there were significantly higher binding indices for monoclonal antibodies to Le^a ($p<0.002$), Le^b ($p<0.005$) and H type 2 antigens ($p<0.001$) on the cell surface of fasting non-smokers compared with cells of fasting smokers. There was significantly higher bacterial binding to cells from 8 non-smokers who were not fasting compared with 8 smokers who were not fasting ($P< 0.002$), and there were significant differences in binding of monoclonal antibodies to H type 2 to cells of non-fasting non-smokers compared with cells of non-fasting smokers ($P< 0.005$). There was no significant difference in binding of the monoclonal antibodies to the Lewis antigens. There was no significant difference in bacterial binding to cells from not-fasting donors compared with cells from fasting donors, binding of anti-H type 2 to cells of non-fasting donors was higher ($p< 0.001$), but there was no significant difference in binding of antibodies to the Lewis antigens (Table 4.4)

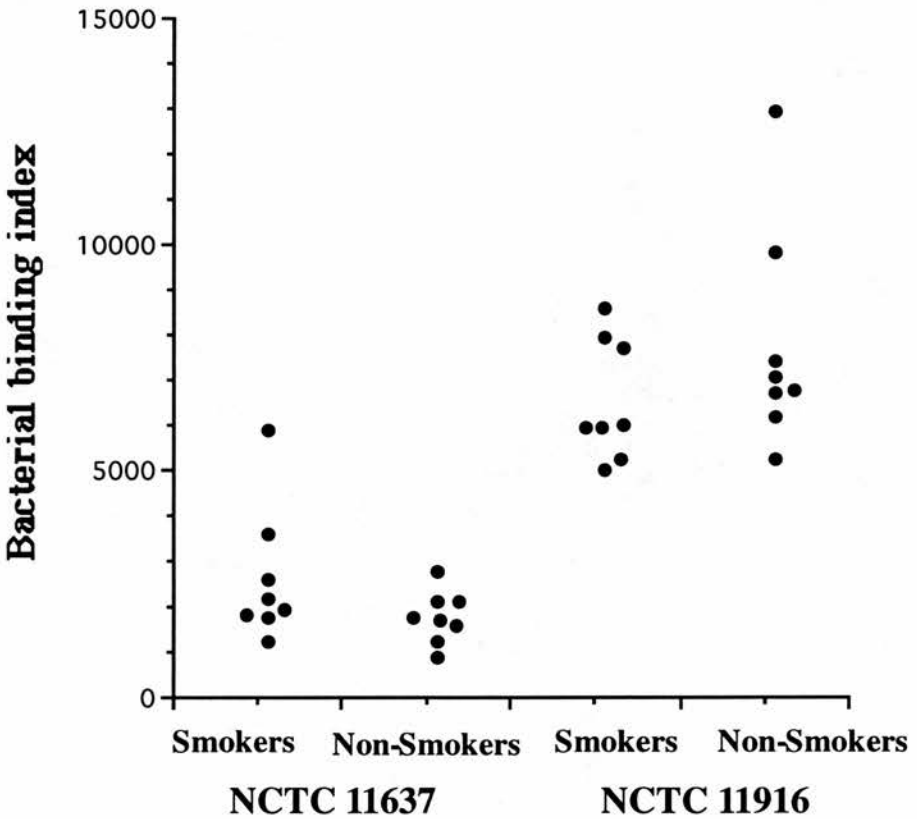


Figure 4.10. Binding of *H. pylori* NCTC 11637 and NCTC 11916 to BEC from healthy students and staff: smokers (n = 8) and non-smokers (n = 8).

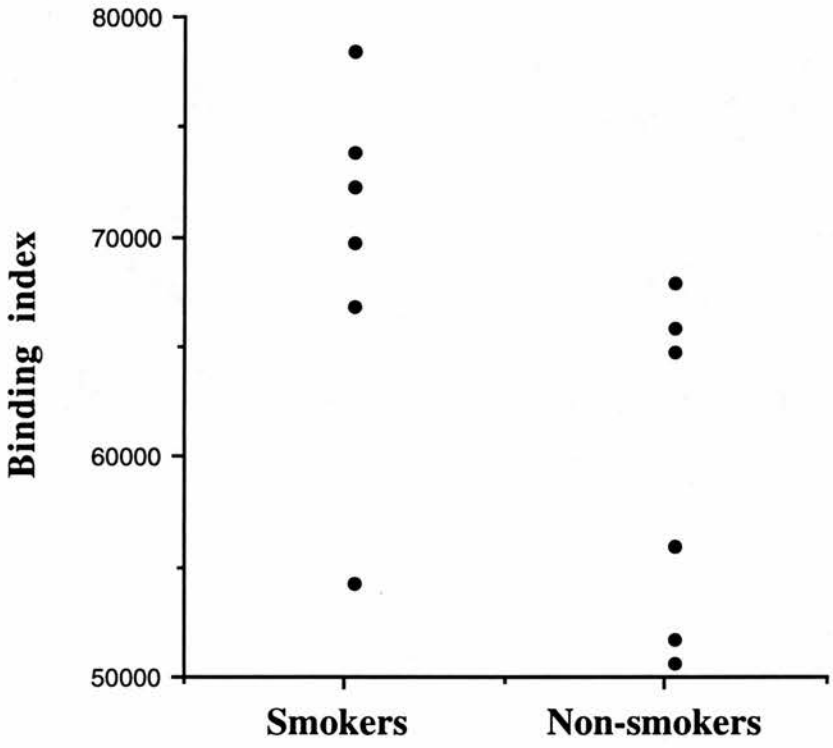


Figure 4.11. Binding of *H. pylori* NCTC 11637 to BEC of 6 smokers and 6 non-smokers referred to the gastroscopy clinic ($P<0.05$).

Table 4.3 Comparison of mean binding indices for *H. pylori* NCTC 11637 and monoclonal antibodies to Le^a, Le^b and H type 2 for BECs obtained from smokers (n = 7) and non-smokers (n = 7) attending gastroscopy clinics.

	Smokers	**Non-smokers**	
	n = 7	**n = 7**	**P**
Bacterial binding	40 301	28 758	0.001
Anti-Le^a	64 721	57 488	NS
Anti-Le^b	23 692	16 811	NS
Anti-H type 2	5 646	2 308	0.02

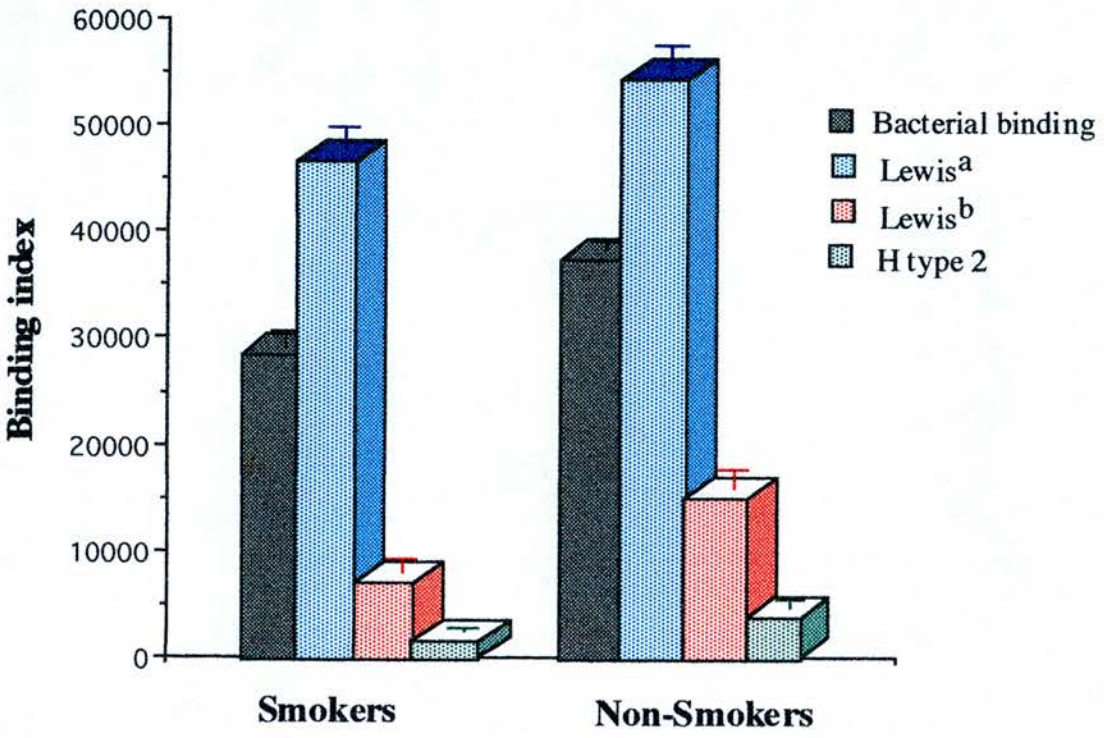


Figure 4.12. Comparison of mean binding indices for *H. pylori* NCTC 11637 and monoclonal antibodies to Le^a, Le^b and H type 2 for smokers (n = 15) and non-smokers (n = 15) of healthy donors.

Table 4.4. Comparison of binding indices for *H. pylori* and monoclonal antibodies specific for H type 2, Le^a and Le^b to buccal epithelial cells from smokers (n = 15) and non-smokers (n = 15) with reference to fasting

	Fasting			**Non-fasting**		
	Smokers	**Non-smokers**		**Smokers**	**Non-smokers**	
	n = 7	n = 7	P*	n = 8	n = 8	P*
H type 2	650	2688	<0.001	2102	4852	<0.005
Lewis^a	47229	58102	<0.002	45359	51031	NS
Lewis^b	7067	17907	<0.005	7902	11869	NS
H. pylori	28815	37299	<0.002	25802	37819	<0.002

* assessed by Rank Sum test

4.3.7 Inhibition of bacterial binding with monoclonal antibodies to H type 2 and Lewis antigens.

In assays with BEC from 12 patients referred for gastroscopy, binding of *H. pylori* strain NCTC 11637 was inhibited with anti-Le^a (28 %), anti-Le^b (25 %) or anti-H type 2 (Serotec) (12 %). The results were analysed by the Wilcoxon Matched-pairs test and there was significant inhibition with monoclonal anti-Le^a ($p<0.02$) and anti-Le^b ($p<0.05$) but not with anti-H type 2 (figure 4.13). Treatment of Kato III cells with different concentrations of monoclonal antibodies against Le^a, Le^b and H type 2 inhibited binding of *H. pylori* NCTC 11637. By the paired t-test, there was no significant difference (figure 4.14).

Assays for inhibition of bacterial binding were also carried out with additional bacterial isolates; two of the isolates were from patients with duodenal ulcers, two isolates were from patients with no histological evidence of disease. Binding of the local isolates and *H. pylori* NCTC 11637 was inhibited by pretreatment of Kato III cells with monoclonal antibodies to H type 2 and Lewis antigens, the mean percentage inhibition was 36 % with anti-Le^a, 35 % with anti-Le^b and 38% with anti-H_2 (figure 4.15). Additive effects on inhibition were observed with combinations of the monoclonal antibodies (figure 4.16)

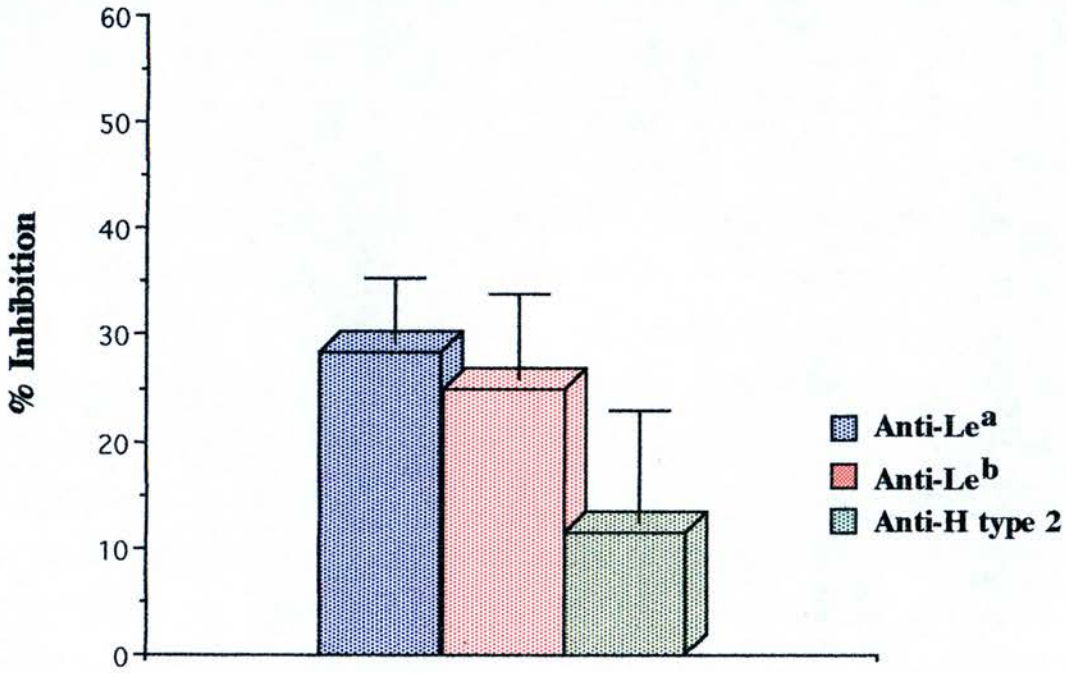


Figure 4.13. Inhibition of bacterial binding of *H. pylori* NCTC 11637 by pretreatment of BEC with monoclonal antibodies against Lewis^a, Lewis^b and H type 2.

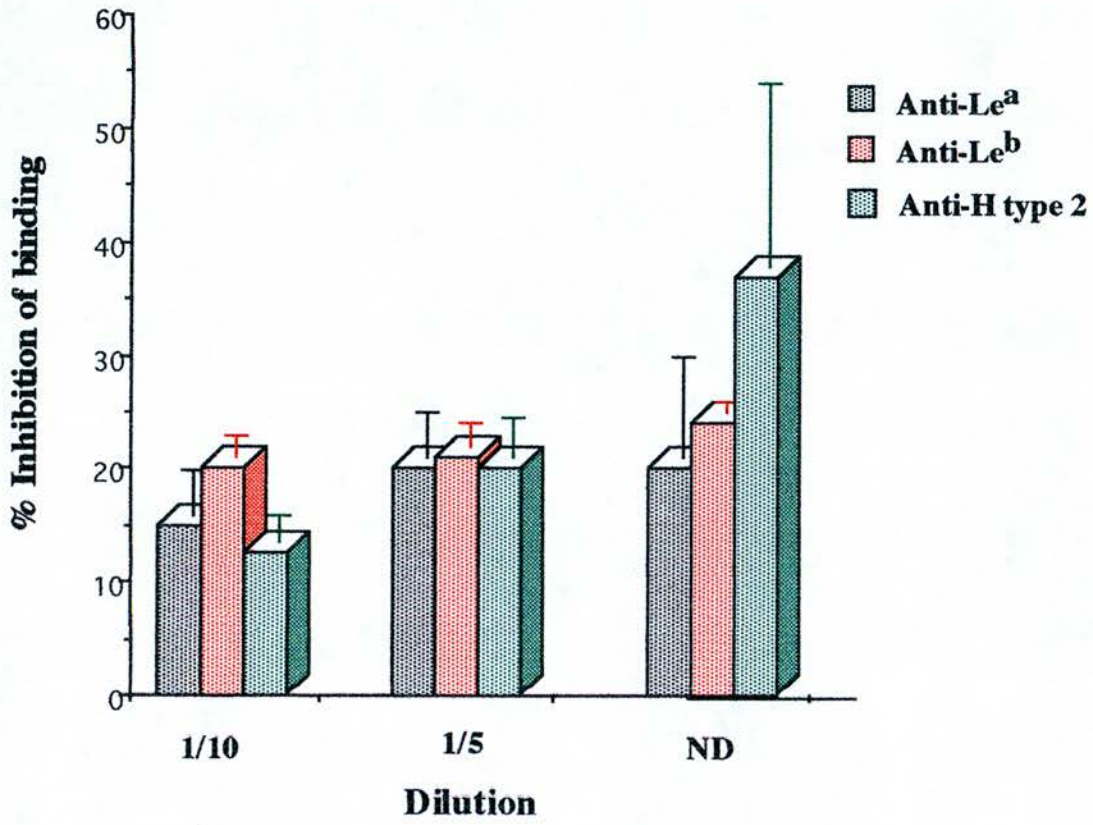


Figure 4.14. Titration of monoclonal antibodies against Le^a, Le^b or H type 2 to assess inhibition of binding of *H. pylori* NCTC 11637 to Kato III cells (ND = not diluted)

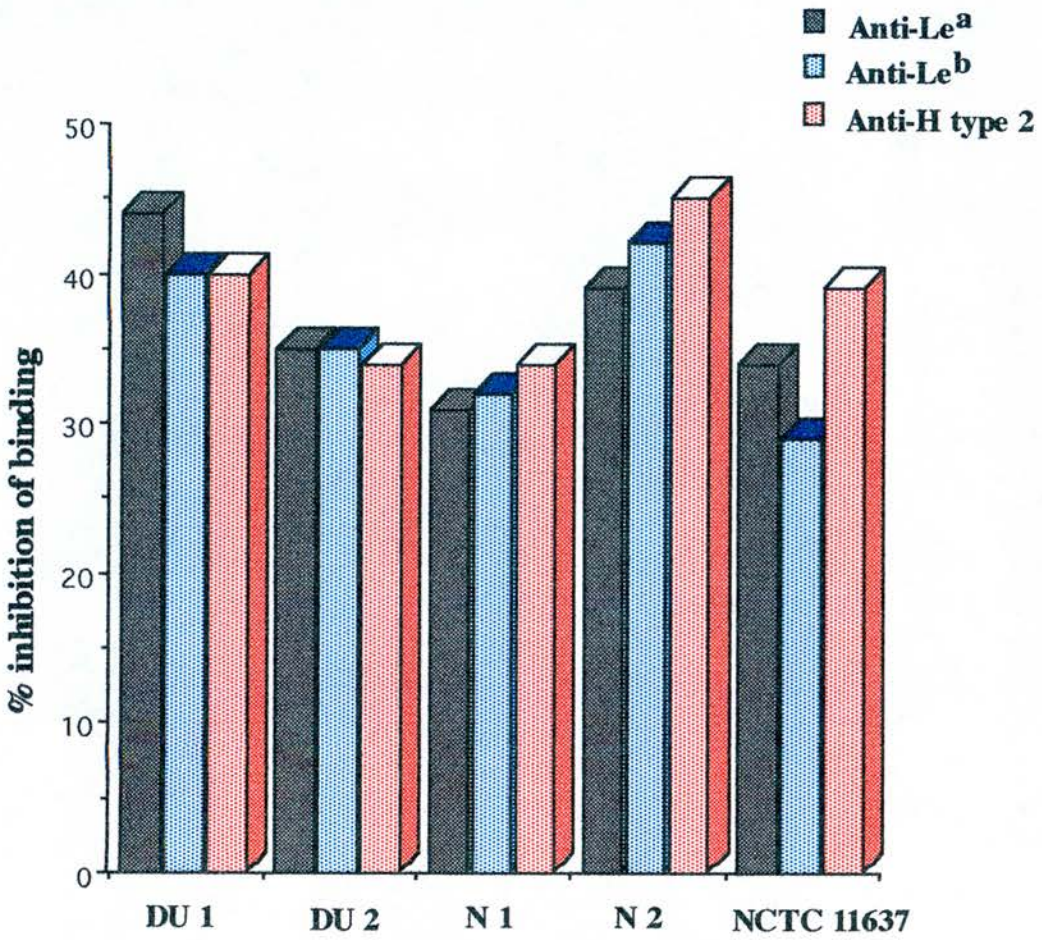


Figure 4.15 Inhibition of bacterial binding of five isolates to Kato III cells preincubated with concentrated monoclonal antibodies against H type 2 and Lewis antigens (DU = isolates from duodenal ulcer patients , N = isolates from patients with no histological evidence of disease

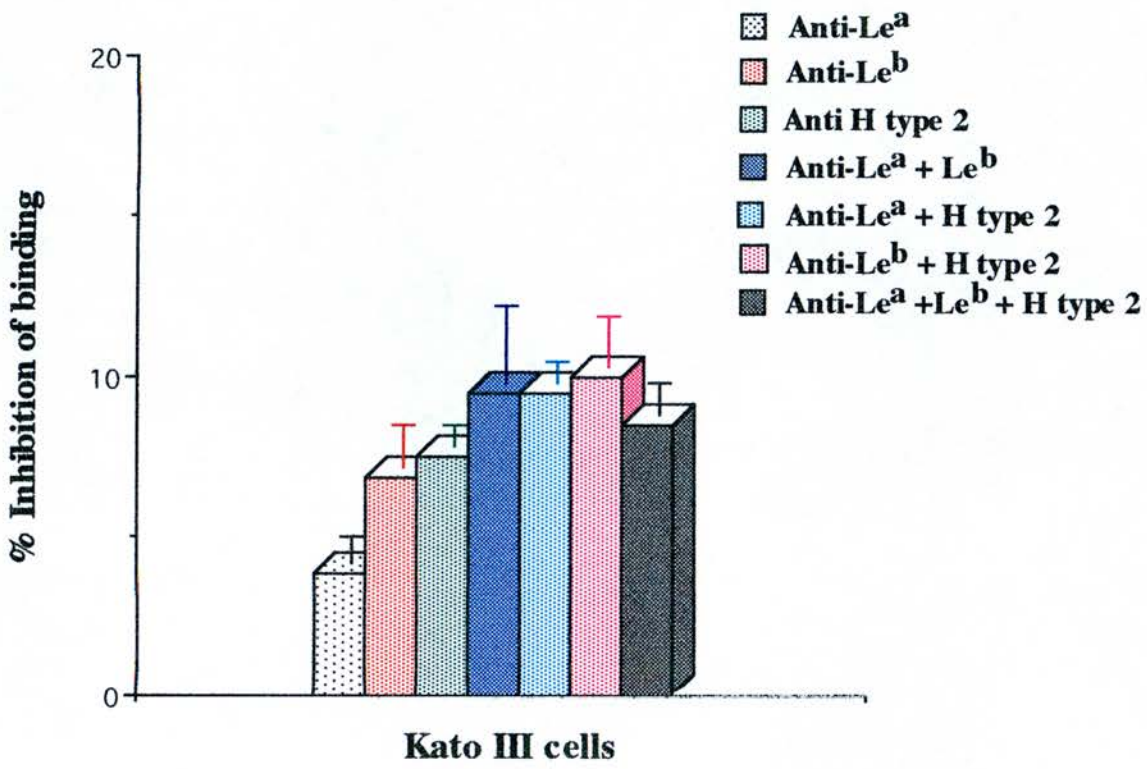


Figure 4.16. Mean of four experiments to assess the inhibition of binding of NCTC 11637 to Kato III cells by pre-treatment of cells with monoclonal antibodies against Le^a, Le^b, H type 2 or combinations of these antibodies

4.4 Discussion

The results of this chapter are assessed in relation to the methodology employed and the objectives set out in the introduction

4.4.1 Methods for assessment of bacterial binding

A number of different techniques have been used to measure the attachment of bacteria to cells. There were several problems associated with the light microscopy method for assessment of bacterial binding, *e.g*, bacteria were not distributed over BEC, it is time consuming to count reasonable numbers of cells and the number of bacteria varied greatly. Comparison between FITC labelled and unlabelled bacteria for binding of *H. pylori* NCTC 11637 to BEC assessed with the light microscopy found no effect of FITC labelling on bacterial binding. The numbers of cells that can be assessed, the accuracy of count and possible bias by the person counting the cells are all factors that limit the use of this method.

Flow cytometry provided a powerful tool for analysing bacterial binding. It was possible to approximate differences in the numbers of bacteria per cell by the changes in the fluorescence intensity of the cells and it was also possible to detect a positive signal from cells even when cells were incubated with smaller numbers of bacteria. It is a reliable and practical method and large numbers of cells can be analysed in a short period of time. Dyes such as fluorescein isothiocynate (FITC) that bind covalently to protein amino groups are preferable to other acid dyes because the washing procedures lower background fluorescence [Shapiro, 1990]. Direct labelling of bacteria with FITC has been used for adhesion assays by other workers [Svenson and Kallenius, 1983; Saadi *et al.*, 1993; Raza *et al.*, 1993], and direct labelling of *Helicobacter pylori* with FITC was used for binding and inhibition studies with human and rat gastric epithelium cells [Falk *et al.*, 1993].

Helicobacter pylori has been shown to bind to a large range of cell types *in vitro*. These cells include human buccal epithelial cells, mouse Y-1 adrenal cells, HEp-2 cells, Int-407 cells, HeLa cells, Kato III cells and primary epithelial cells isolated from gastric, duodenal and colonic biopsy specimens [summarised by Clyne and Drumm, 1993]. BEC were chosen for the binding assays because of their ready availability and the expression of blood group antigens under investigation on their cell surfaces. Although many cells are dead and there are variations in size of the cells, they have been used for binding assays with many bacterial species: type II Group-B *Streptococcus pyogenes* [Bagg, 1982], *Neisseria meningitides* [Craven and Frasch, 1978], *Streptococcus pneumonia* [Mahajan and Panhotra, 1989] and *Staphylococcus aureus* [Beck, 1989; Saadi *et al*, 1993].

4.4.2 Expression of proposed receptors on BEC, gastric cells and Kato III cells

The first objective of the study was to determine if the blood group antigens proposed to act as receptors for *H. pylori* were present on gastric mucosa, BEC and Kato III cells to be used in binding and inhibition studies. The small numbers of mucosal cells obtained from gastroscopies precluded their use in the bacterial binding assays. Monoclonal antibodies against H type 2, Le^a or Le^b bound to each of the cell types tested. The binding of anti-Le^b to cells from non-secretors observed in this study (Table 4.2) was probably due to the cross reactivity of anti-Le^b with both Le^a and with H related structures [Good *et al.*, 1992]. Previous studies of human gastric carcinoma and normal gastric tissue has demonstrated blood group antigens by immunohistochemical methods [Sakamoto *et al.*, 1989], and the present study confirmed the expression of these blood group antigens on the cell surface of normal gastric tissue and gastric adenocarcinoma cell line by the flow cytometry technique.

There was no difference in expression of these antigens between males and females; therefore, BEC from 30 healthy male donors were used to assess the effects of smoking and fasting on expression of these antigens. Fasting significantly reduced binding of the monoclonal antibody to H type 2 but not binding of monoclonal antibodies against Lewis antigens. Binding indices for all three monoclonal antibodies were significantly lower for cells from smokers compared with non-smokers. This might be due to components of the cigarette smoke covering the antigen on epithelial cell surfaces.

4.4.3 Role of blood group antigens as receptors for *H. pylori*

The second objective was to determine if in addition to Le^b previously identified as a receptor for *H. pylori* [Borén *et al.*, 1993], Le^a or H type 2 could also act as receptors for the bacteria. Borén *et al* [1993] found no strong evidence for the involvement of fucose-containing blood group antigens other than $Lewis^b$ in binding of *H. pylori* in their model system [Borén *et al.*, 1993]. In contrast, the results of this chapter indicate that antibodies to H type 2, Le^a or Le^b were capable of inhibiting *H. pylori* binding to Kato III and BEC. This might be due to different methods as Borén *et al* used tissue sections and assessed binding microscopically. This study examined cells in suspension and detected binding by flow cytometry. Different monoclonal antibodies were used in the two studies, and one of the monoclonal antibodies to H type 2 (SAPU) did not inhibit binding in these studies.

The results of the bacterial binding studies with BECs from 30 individuals indicated that the binding indices for *H. pylori* were significantly associated with binding indices for monoclonal antibodies to H type 2 ($P< 0.005$) and Le^b ($P< 0.001$) but not with Le^a. Pretreatment of Kato III or BECs with the monoclonal antibodies to each of the three blood group antigens reduced the binding of *H. pylori* . For the Kato III cells, the binding indices for anti-Le^a were higher than for the other two antibodies

but inhibition of bacterial binding was greatest with anti-H type 2 followed by anti-Le^b with the lowest inhibitory effect noted for anti-Le^a. For BECs from patients referred for gastroscopy, the inhibition of bacterial binding was greatest with Le^a followed by Le^b then H type 2. These different inhibition results might be due to the amount of blood group antigens expressed on the cell surface of different cells.

These results suggested that blood group antigens (H type 2, Le^a and Le^b) might play an important role in attachment of *H. pylori* to the gastric mucosa. In each set of experiments with BEC, binding indices of *H. pylori* was correlated with binding indices for H type 2, indicating that H type 2 found on almost all individuals is a key receptor for *H. pylori*. The increased susceptibility of group O individuals to peptic ulcer disease might be due partly to higher density of colonisation by these bacteria compared with other blood groups.

While both secretors and non-secretors express H type 2, these results indicate the higher levels of Le^b in mucus of secretors might compete more effectively for the adhesin than Le^a, thereby reducing density of colonisation among secretors. The terminal fucose structure present on blood group antigens found in body fluid of secretors as in the H type 1 structure found in $Lewis^b$, A-$Lewis^b$ or B-$Lewis^b$ might bind to the adhesin and reduce colonisation.

4.4.4 The effects of smoking and fasting on bacterial binding and expression of blood group antigens

Smokers have been shown to have enhanced adherence of *Strept. pneumonia* to BEC [Mahajan and Panhotra, 1989; Raman *et al.*, 1983; El-Ahmer *et al.*, 1996], and smokers with chronic bronchitis appear to have increased binding of *H. influenzae* to their pharyngeal cells [Fainstein and Musher, 1979]. While smoking has been identified as a risk factor for ulcers, the present study with BEC suggest that in healthy individuals smoking does not result in increased expression of H type 2 or

Lewis blood group antigens. If there are similar effects on epithelial cells lining the gastric mucosa, smoking would not be expected to enhance bacterial binding. Both sets of experiments with cells from patients found higher bacterial binding to cells of smokers. There were also increased levels of binding of anti-H type 2 to the cells of smokers in the second group, this led to examination of the effects of fasting on bacterial binding in relation to smoking.

Fasting is one of the environmental factors associated with peptic ulcers. Dönderici and colleagues noted that peptic ulcer complications were higher during Ramadan than the periods before and after the fast [Dönderici *et al.*, 1994]. It has been claimed that long term fasting may contribute to healing of persistent ulcers by improving the control of stomach secretion [Johnston and Wormsley, 1989]. Collection of BEC samples during the month of Ramadan provided an opportunity to study the effect of fasting on binding of *H. pylori* to BECs in a non-patient group. Fasting did not enhance bacterial binding or expression of Lewis antigens but there were significantly higher levels of H type 2 for non-fasting donors compared cells obtained when they were fasting. This could be related to lower saliva output associated with fasting which is in agreement with previous observations in which deprivation of salivary epidermal growth factor (EGF) significantly lowered levels of salivary carbohydrate, lipids and covalently bound fatty acids, but showed higher levels of protein content than normal [Sarosiek *et al.*, 1988].

4.4.5 Conclusions

The evidence in this chapter indicated H type 2, Le^a and Le^b can act as receptors for *H. pylori* adhesion; therefore, the next step was to employ the affinity purification method with synthetic blood group antigens [Saadi *et al.*, 1994] to attempt to obtain adhesins from *H. pylori* that bind H type 2 and Lewis antigens.

Chapter 5

Isolation of a cell surface component of *H. pylori* that binds to H type 2, Lewis^a and Lewis^b blood group antigens.

5.1 Introduction

Several surface components thought to be involved in binding of *H. pylori* to epithelial cells have been identified (Table 5.1). While Le^b has been proposed to act as a receptor for these bacteria [Borén *et al.*, 1993], the surface component(s) that bind to the antigen has not been identified. The results presented in Chapter 4 found binding of *H. pylori* to Kato III or BEC could be inhibited by pretreatment with monoclonal antibodies against H type 2, Le^a or Le^b and that binding of *H. pylori* correlated significantly with binding of monoclonal antibodies to H type 2 and Le^b. Both contain a terminal fucose in their structures which is not present on Le^a (Figure 1.3).

Studies in which inhibition of bacterial binding by antibodies to the host cell antigens is demonstrated are open to the criticism that the antibodies are binding to an epitope near the site of the actual receptor, the inhibition observed being due to stearic hindrance. Two methods were proposed to obtain additional evidence that H type 2 and the Lewis antigens are receptors for *H. pylori*: direct binding of synthetic blood group antigens (now available at a reasonable cost) to the bacteria; and synthetic Le^a has been used for affinity purification of adhesin(s) from *S. aureus* [Saadi *et al.*, 1994].

The aims of this section were: 1) to develop a direct attachment assay for binding of biotinylated blood group antigens to whole cells of *H. pylori*; 2) to isolate surface components of *H. pylori* that bind H type 2 or Lewis blood group antigens by affinity purification; 3) to assess the ability of the component(s) obtained by affinity purification to reduce attachment of the bacteria to epithelial cells; 4) to determine what proportion of *H. pylori* isolated from local patients expressed the adhesin(s) that binds the H type 2 and Lewis blood group antigens.

Table 5.1

Putative adhesins of *H. pylori* and their receptors

Adhesin	Receptor	References
20 kDa	Haemagglutinin NeuAc-lactose specific	Evans *et al.*, 1988 and 1993
25 kDa	Haemagglutinin NeuAc-lactose specific	Huang *et al.*, 1992
59 kDa	Haemagglutinin specificity unknown	Huang *et al.*, 1992
63 kDa	Phosphatidylethanolamine	Lingwood *et al.*, 1993
60	Hela cells specificity unknown	Fauchere and Blaser., 1990
Unknown	Le^b blood group	Borén *et al.*, 1993

5.2 Materials and Methods

5.2.1 Bacteria Strains and growth culture

H. pylori NCTC 11637 and 51 local patient isolates were cultured for 5 days under the conditions described in (2.8.1).

5.2.2 Extraction of outer membranes

H. pylori NCTC 11637 was cultured on Campylobacter selective medium as described (2.6.1). Outer membranes (OM) were obtained by the method of Blaser *et al.* [1983]. Bacterial suspensions were washed 3 times in 0.01M Tris buffer (pH 7.4) then sonicated for 15 min (three 5 minutes intervals with a period for cooling between each). The cells were centrifuged 1000 x *g* for 20 min. The supernatant was collected and centrifuged at 100,000 x *g* for 60 min at 4°C and the resulting pellet was treated with 5 ml of 1% sodium N-lauroylsarcosine solution (Sarkosyl) and incubated for 20 min at 37°C. OM representing the Sarkosyl-insoluble fraction was collected by centrifugation at 50,000 x *g* for 60 min at 4°C, resuspended in sterile distilled water, freeze dried and stored at -20°C.

5.2.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) of OM

Outer membrane proteins (OMP) were separated by SDS-PAGE using the SDS-discontinuous system with a Biometra-Minigel (Biometra, Wagen-Stieg, Göttingen, Germany). Equal volumes of protein sample and sample buffer were mixed and heated to 100°C for 5 min. Samples were applied to each lane and electrophoresis performed at a constant voltage of 40 V through the stacking gel (5% acrylamide) and at a constant voltage of 70 V through the resolving gel (10% acrylamide). OMPs were visualised by staining with Coomassie brilliant blue (0.5%, w/v) in 25%

(v/v) propan-2-ol + 10% (v/v) glacial acetic acid followed by destaning with 10% (v/v) glacial acetic acid.

5.2.4 Purification of OMP by affinity adsorption

Synsorb affinity adsorbent (1 mg/ml) (Chem Biomed Ltd., Edmonton, Alberta, Canada; lot ASi-137) with Le^a, Le^b or H type 2 covalently linked to the silica matrix was swollen in universal containers in 2 ml of PBS (pH 7.2) for 2 h at room temperature with continuous rotation. The beads were centrifuged at 50 x *g* for 5 min and the supernatant removed. The OMP extracted from strain NCTC 11637 (65 µg/ml) was added to the beads and incubated with continuous rotation overnight at 4°C then centrifuged at 50 x *g* for 5 min and the supernatant containing unbound material was collected. The beads were washed three times with 5 ml of PBS and the washings added to the unbound material. The bound material was eluted from Synsorb beads by 2 ml of 2% ammonia in saline for 20 min with continuous rotation at room temperature. The supernatant was recovered following centrifugation at 50 x *g* for 5 min. The beads were washed with 5 ml of PBS and centrifuged again. The combined eluates were freeze dried and reconstituted in 300 µl distilled water (Figure 5.1).

5.2.5 Dot blot assay

5.2.5.1 High salt buffer (HSB)

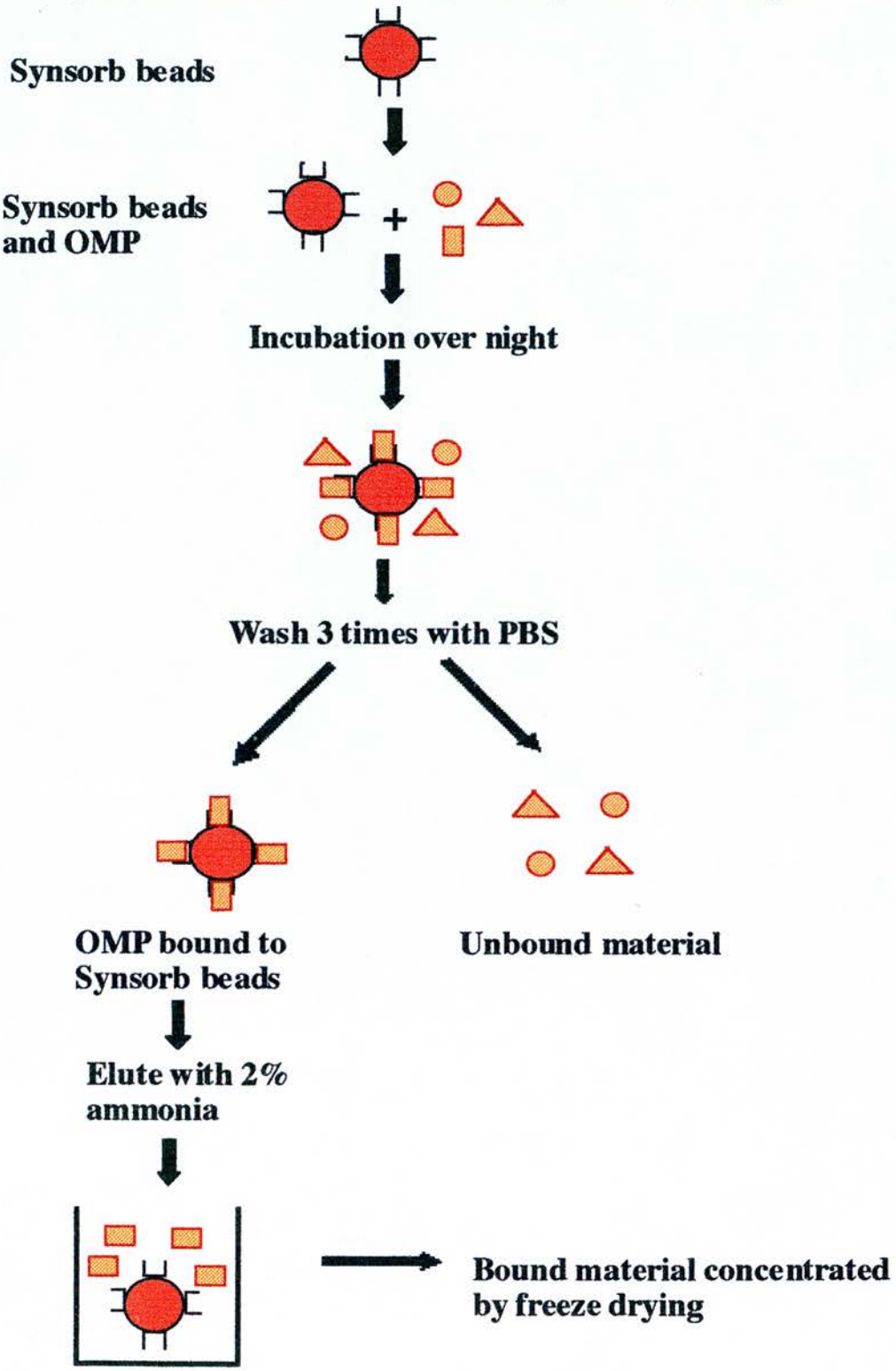
HSB was composed of PBS containing 1M NaCl and Tween 20 (0.5%, v/v) (DBH, Poole, Dorset).

5.2.5.2 Substrate

The substrate contained 160 mg dioctyl sulfo-succinate (DONS) (Sigma), 49 mg tetramethyl benzidine (TMB) in 12 ml of buffer composed of 24.3 ml 0.1 M citric

acid (BDH), 25.7 ml 0.2 M Na_2HPO_4 (BDH), 50 ml ethanol and 60 ml distilled water. The mixture was placed in a water bath at 60°C for 30 min and activated by adding 60 µl hydrogen peroxide (30% v/v) (Sigma) immediately prior to use.

Figure 5.1 Purification of adhesin by affinity adsorption



5.2.6 Dot blot of whole bacteria

A heavy bacterial suspension of *H. pylori* strain NCTC 11637 was made by transferring several colonies with a wire loop from a subculture plate to a tube containing 200 µl PBS and heated at 100°C for 60 min to inactivate catalase. The nitrocellulose membrane (Gelman Sciences, Northampton) was cut into rectangles to fit into split plates. Circles were drawn in pencil on the shiny side of the membrane. The bacterial suspension (10 µl) was added to circles on rectangular strips of each membrane which were to receive different treatments. As positive controls monoclonal antibodies to Le^a, Le^b, $Lewis^x$ (Le^x) (Serotec) and H type 2 were added to the strips without bacteria in a similar manner. The strips were incubated for 10 - 15 min at 37°C. Biotinylated Le^a, Le^b , Le^x or H type 2 (10 µl) (Syntesome GmBh, Fine Biochemicals) diluted in HSB (100 µg ml^{-1}) were added to separate circles and incubated at 37°C for 1 hr. A washing stage in HSB followed for 15 min and the fluid was discarded. The strips were immersed in tap water for 1 min and placed in HSB. HSB was discarded and streptavidin horseradish peroxidase (HRP) (Sigma) (0.5 ml, 1.3 µg ml^{-1}) was added and incubated at 37°C for 1 hr. After a final wash in HSB, 2 ml of the substrate (5.2.4.2) was added and incubated with the strips at room temperature for 5 min for colour development. The reaction was stopped by washing with tap water.

5.2.7 Dot blot of outer membrane proteins

The same method as described in (5.2.6) was used for the outer membranes.

5.2.8 Binding of biotinylated blood group antigens to whole bacteria

An assay for detection of biotinylated blood group antigens to whole cells of *H. pylori* was developed. Bacteria were grown for 5 days under the conditions described earlier (2.6.1). A heavy suspension in PBS was heated at 100°C for 60

min then centrifuged at 1000 x *g* for 20 min. The pellet was resuspended in coating buffer (2.1.1) and standardised by optical density to a total count of 3.2 x 10^8 determined by correlation of OD at 540 nm with direct microscopy (2.6.3).

Batches of 96 well microtitre plates were coated with 100 µl of bacteria and incubated overnight at 4°C. The plates were washed with washing buffer (2.1.3), blocked with blocking buffer (2.1.4) and incubated at room temperature for 20 min. The blocking buffer was removed and the plates washed with washing buffer.

Biotinylated Le^a, Le^b, Le^x or H type 2 antigens diluted in blocking buffer to 100 µg ml^{-1} were added to the wells and incubated at 37°C for 60 min. The plates were then washed 6 times with washing buffer. Streptavidin-HRP (100 µl, diluted to 1.3 µg ml^{-1} in blocking buffer) (Sigma) was added to the plates and incubated for 60 min at room temperature then washed 6 times in washing buffer. The substrate (100 µl) (2.1.6) was activated by 40 ml H_2O_2 (30% v/v) immediately before use and added to each well. The colour change was stopped after 10 - 20 min by adding 100 µl of H_2SO_4 (2.1.7) (Fig 5.2). The optical density at 490 nm was determined by an ELISA reader (Dynatech) and corrected by subtracting the OD of the corresponding blank well containing bacteria, streptavidin-HRP and substrate.

5.2.9 Binding of biotinylated blood group antigens to the component isolated by affinity adsorption

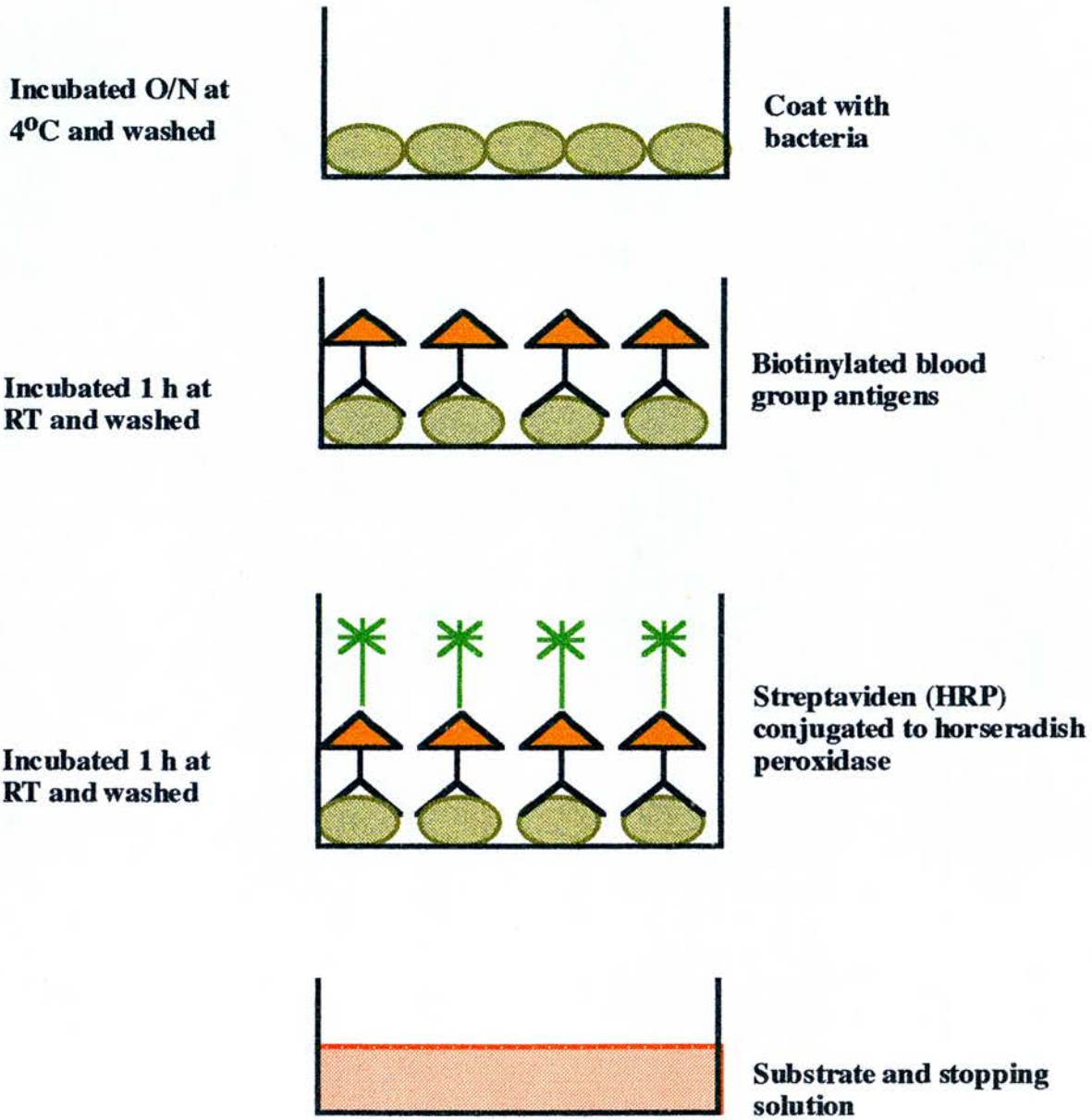
An assay for detection of binding of biotinylated blood group antigens to affinity purified protein was developed. The same protein concentrations of unbound supernatant and the proteins eluted from the Synsorb beads were used to coat wells of a 96 well microtitre plate (100 µl of 10 µg ml^{-1} protein) and incubated overnight at 4°C. The plates were treated as described above (5.2.7).

5.2.10 Inhibition of binding of *H. pylori* to Kato III cells

A heavy suspension of the bacteria was prepared in PBS and washed three times by centrifugation at 1000 x *g* for 20 min. The bacteria were labelled with FITC and adjusted to 3.2 x 10^8 as described in 4.2.2.

The material eluted from Synsorb H type 2, Le^a or Le^b (adhesin) was resuspended in PBS and 200 µl was added to 200 µl of the Kato III cell suspension . The cells were incubated at room temperature for 60 min, washed twice in PBS by centrifugation at 600 x *g* for 10 min and the labelled bacteria (200 µl, 300 bacteria /cell) added to each sample. Controls were Kato III cells incubated with PBS but no protein eluate. The samples were incubated at 37°C for 60 min with gentle shaking (100 rpm) in an orbital incubator (Gallenkamp) then washed twice in PBS at 600 x *g* for 10 min. The washed cells were resuspended in 150 µl of PBS and fixed with 100 µl of 1% buffered paraformaldehyde. They were stored in the dark at 4°C until analysed by flow cytometery (Coulter EPICS-XL, UK). The binding index (BI) for each sample was calculated from the percentage of the cells with fluorescence greater than the background multiplied by the mean fluorescence of the positive population. The percent inhibition was calculated by the formula used in chapter 4.

Figure 5.2 Attachment of biotinylated blood group antigens to *H. pylori*



O/N = over night **RT = room temperature**

5.3 Results

5.3.1 Dot blot of whole cells bacteria and OM

Dot blot assays with whole cells of *H. pylori* NCTC 11637 or equivalent amounts of the OM extract appeared to bind more biotinylated H type 2 as judged by the colour intensity of the blot. Le^a appeared to bind the least and Le^b gave an intermediate colour. No colour was observed with biotinylated $Lewis^x$. The quality of the photographs were not sufficient to demonstrate the differences observed; therefore a quantitative spectrophotometric assay was developed.

5.3.2 Binding of biotinylated blood group antigens to *H. pylori*

Direct attachment of biotinylated blood group antigens to *H. pylori* NCTC 11637 was examined with different concentration of bacteria 3.5, 1.75, 0.87 and 0.44 x 10^8. Figure 5.3 showed the dose response effect of bacterial concentration on binding of the blood group antigens. Further experiments were carried out with bacteria adjusted to 3.2 x 10^8.

Figure 5.4 shows the mean of six experiments in which binding of biotinylated blood group antigens to *H. pylori* strain NCTC 11637 was assessed in microtitre plates. The highest optical density readings were observed with H type 2 followed by Le^b and the lowest values with Le^a. By the t-test for two groups test, OD values for H type 2 was significantly higher than Le^b ($P< 0.02$), H type 2 was significantly higher than Le^a ($P< 0.003$), Le^b significantly higher than Le^a ($P< 0.002$), H type 2 was significantly higher than group A and group B ($p < 0.02$ and $p < 0.03$ respectively) and Le^b was significantly higher than group A and group B ($p < 0.03$ and $p < 0.01$ respectively) Sequential culture (5 subcultures) of the strain did not alter the pattern of binding of the blood group antigens.

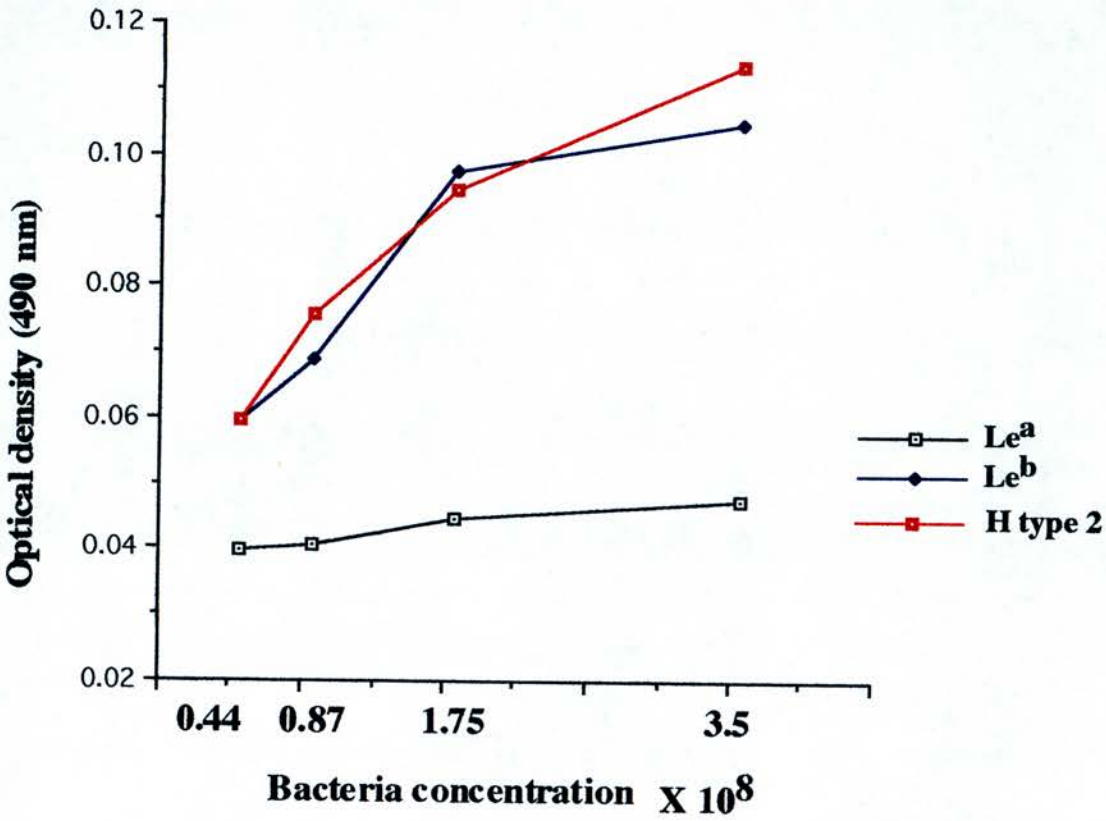


Figure 5.3 Dose response of bacterial binding to biotinylated blood group antigens

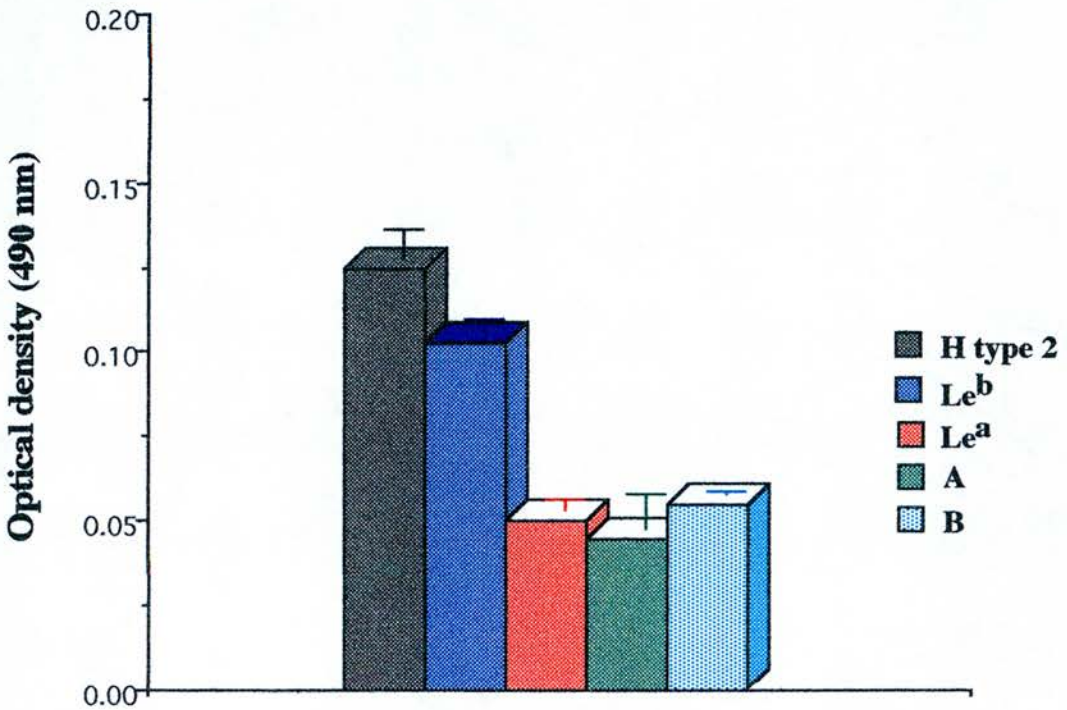


Figure 5.4. Mean of six experiments to assess binding of biotinylated blood group antigens to *H. pylori* NCTC 11637.

5.3.2.2 Binding of biotinylated blood group antigens to OMP

Attachment of the blood group antigens to OMP extracted from *H. pylori* NCTC 11637 was assessed by the same assay with 4 different concentration of OMP 5, 2.5, 1.25 and 0.6 μg ml^{-1}. The results showed a dose dependent response; the highest optical density readings were observed at 5 μg ml^{-1} protein. The highest optical density readings were observed with H type 2 followed by Le^b and the lowest values with Le^a (Figure 5.5).

5.3.3 Isolation of the adhesin by affinity adsorption

SDS-PAGE (10% acrylamide) of the Sarkosyl-insoluble outer membrane protein of NCTC 11637 stained with Coomassie blue showed three major bands migrating at approximately 30, 45 and 61 kDa; additional bands were seen at 54, 75, 110 and 142 kDa. The protein concentration of the original OMP extract was 65 μg ml^{-1} (track 1). The protein concentrations of the material eluted from Synsorb H type 2, Le^a and Le^b were 15 μg ml^{-1}, 5 μg ml^{-1} and 10 μg ml^{-1} respectively. The material eluted from Synsorb H type 2, Synsorb Le^a and Synsorb Le^b migrated as a band at approximately 61 kDa (figure 5.6).

5.3.4 Inhibition of bacterial binding by the adhesin

Binding of *H. pylori* NCTC 11637 to Kato III cells was inhibited with the material eluted from Synsorb H type 2, Le^a or Le^b. The percentage inhibition of bacterial binding was 74% with the material eluted from Synsorb H type 2, 64% material eluted from Le^a and 67% with material eluted from Le^b (figure 5.7). This reflected the protein concentration of the individual elutes.

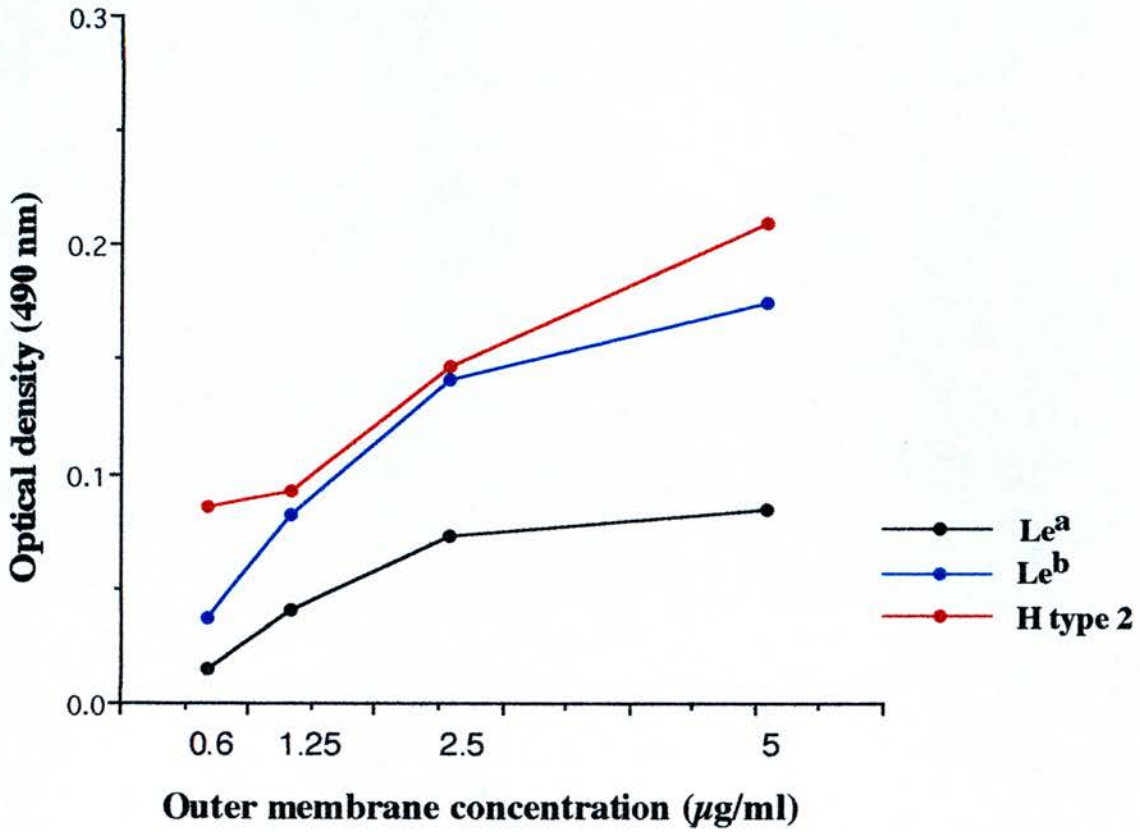


Figure 5.5 Dose response of binding of outer membrane protein to biotinylated blood group antigens assessed by microtitre plate assay.

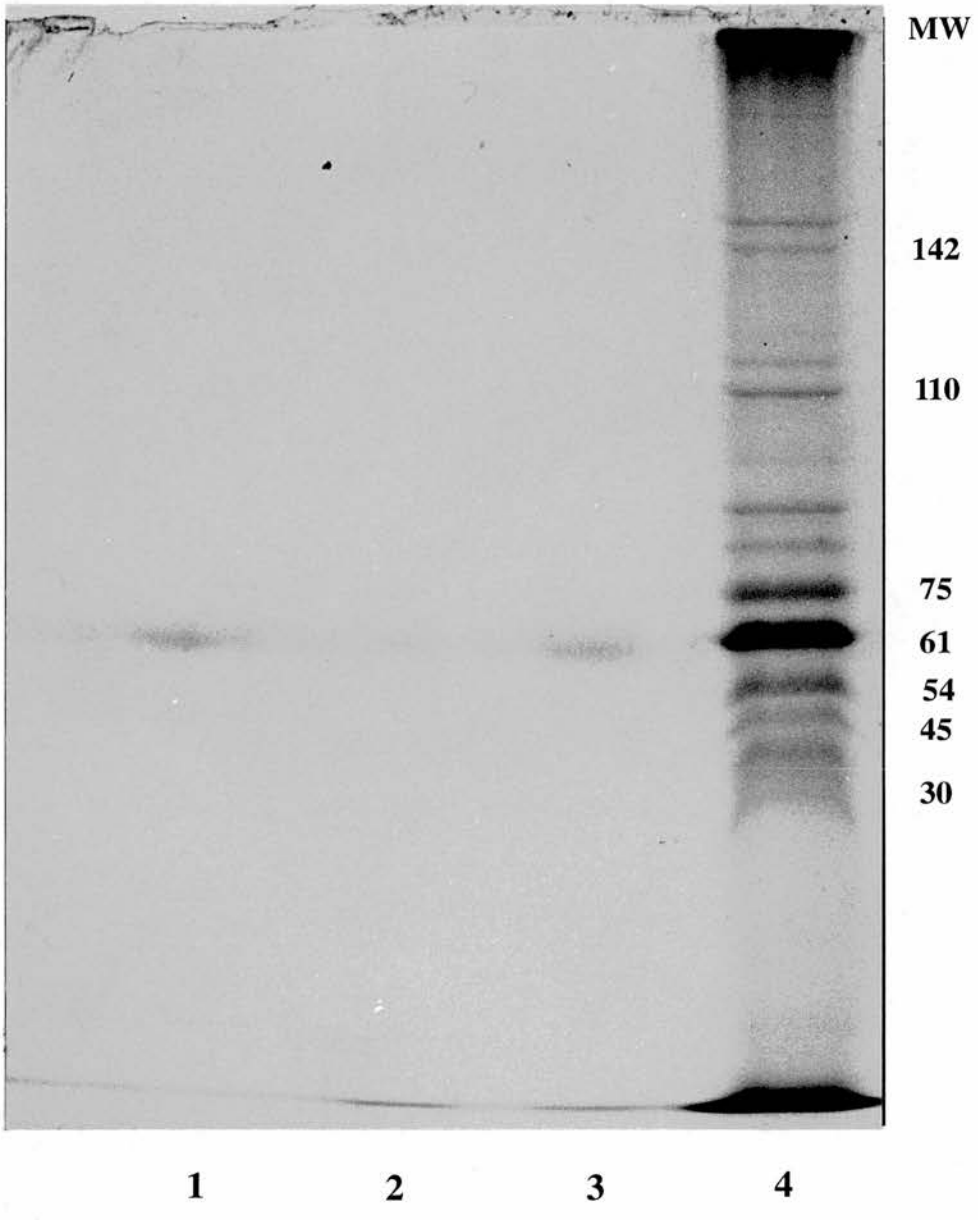


Figure 5.6 Coomassie brilliant blue stained SDS-PAGE gel (10%) of Sarkosyl insoluble membrane (track 4), material eluted from Synsorb Lewis^b (track 3), Synsorb Lewis^a (track 2) and Synsorb H type 2 (track 1).

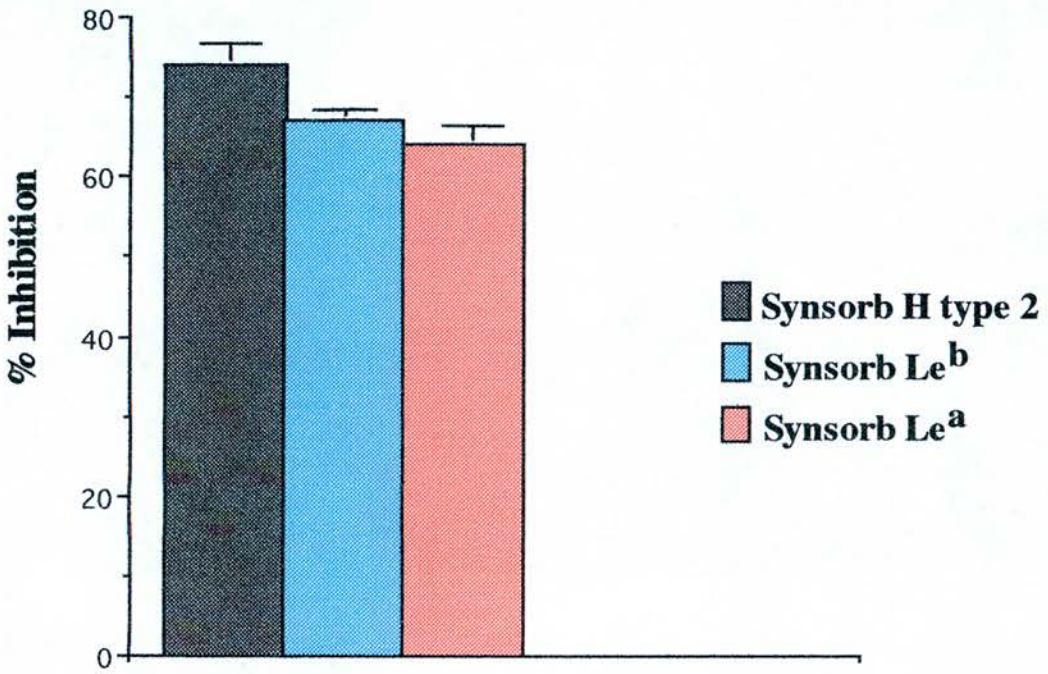


Figure 5.7 Inhibition of binding of *H. pylori* NCTC 11637 to Kato III cells following treatment with material eluted from Synsorb beads (mean of 4 experiments)

5.3.5 Binding of biotinylated blood group antigens to the adhesin isolated by affinity adsorption

Binding of the biotinylated blood group antigens to OMP, purified adhesin and unbound material was compared at the same protein concentration (10 μg ml^{-1}) by the spectrophotometric assay. The same patterns were obtained with dot blot and microtiter assays; H type 2 gave the highest OD or colour intensity, Le^b was intermediate and Le^a the lowest. The purified adhesin bound higher levels of the blood group antigens compared with OMP and the lowest values were observed with unbound material at the same protein concentration (figure 5.8).

5.3.6 Reproducibility of test

The 51 local patient isolates were tested on 2 occasions, strain NCTC 11637 was included in each experiment as a control and Le^x was used as negative control. All isolates bound H type 2, Le^b and Le^a but not Le^x.

5.3.7 Patterns of binding of biotinylated blood group antigens to local *H. pylori* isolates

Among the 51 patient isolates, there were four patterns of attachment of the biotinylated oligosaccharides (Table 5.2). For the majority of the strains, the pattern was similar to that of NCTC 11637, the highest OD values were observed with H type 2, the lowest with Le^a and Le^b was intermediate. There were 5 isolates for which the OD values obtained with Le^a were greater than or equal to those for Le^b and there were 3 isolates for which binding of Le^a and Le^b were equivalent.

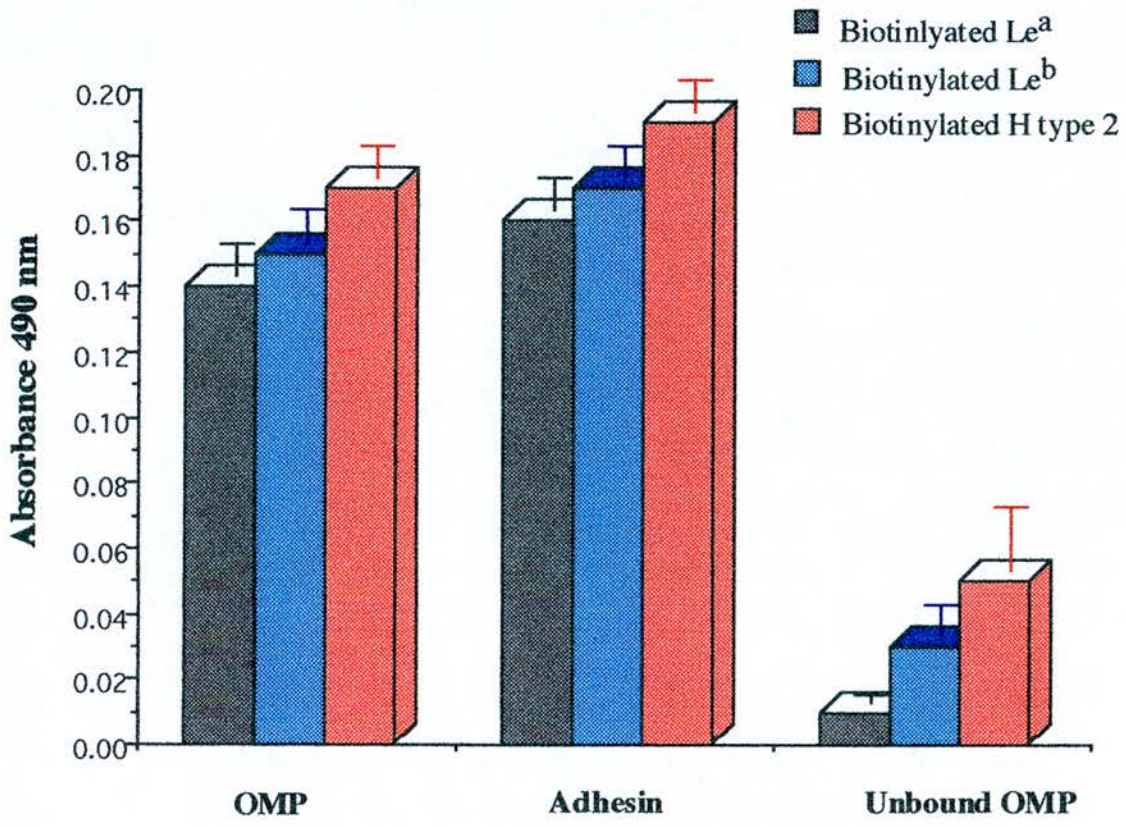


Figure 5.8 Mean of two experiments of to detect binding of biotinylated blood group antigens to adhesin of *H. pylori* NCTC 11637 obtained by synthetic Synsorb beads compared with equivalent concentration (10 μg ml^{-1}) of OMP and unbound materials.

Table 5.2 Binding of biotinylated blood group antigens to *H. pylori* isolated from patients attending the gastroenterology clinic.

ABH antigens	***H. pylori* isolates n = 51**	**%**	**Blood group O**		**Non-secretor**	
			n	**%**	**n**	**%**
H2 > Le^b > Le^a	37	72.5	27/37	72.9	17/37	45.9
Le^b > H2 > Le^a	7	13.7	4/7	57	3/7	42.8
Le^a > Le^b	3	5.9	1/3	33.3	2/3	66.6
Le^a = Le^b	4	7.8	0/4	0	2/4	50

5.4 Discussion

5.4.1 Development of an assay for direct binding of blood group antigens to *H. pylori*

The first aim of this part of the study was to develop a method to demonstrate direct binding of the blood group antigens proposed to act as receptors for *H. pylori*. The microtitre spectrophotometric assay was developed to provide a rapid, quantitative and reliable method for screening isolates to determine what proportion of them bound the blood group antigens. All steps and working concentrations were adjusted to obtain the maximum reading while keeping the OD values for negative controls low, including the optimal bacteria concentration in the coating buffer which leads to the formation of a monolayer of bacteria on the surface of the wells. The technique for coating the bacteria is easy to perform and to standardise. The method can be applied to a wide range of *H. pylori* isolates. The OD values complemented the colour intensity observed in the dot blot assay which could not be quantified.

5.4.2 Attachment of biotinylated blood group antigens to *H. pylori*

All the 51 patient isolates tested bound H type 2, Le^a, Le^b but not Le^x, 4 patterns were observed. The majority (72%) were similar to that of NCTC 11637 H type 2 > Le^b > Le^a. There was no significant association with disease, ABO group or secretor status. The majority of the isolates exhibited a binding pattern similar to that of NCTC 11637. This suggests that all the strains tested express the adhesin that binds the fucose containing blood group antigens.

5.4.3 Isolation of a 61kDa protein by affinity purification

The results in chapter 4 indicated that the putative receptors H type 2, Le^a and Le^b are present on the gastric cells. The SDS-PAGE analysis of the OMPs of *H. pylori*

NCTC 11637 are in agreement with previous findings by other groups; there were three major OMPs bands at approximately 61, 54 and 30 kDa [Newell., 1987; Czinn *et al.*, 1989 and Drouet *et al.*, 1991]. The 61 kDa protein was obtained by affinity adsorption with synthetic Synsorb H type 2, Le^a or Le^b with the greatest amount eluted from H type 2, the lowest from Le^a and an intermediate amount from Le^b.

5.4.4 Inhibition of bacterial binding by pretreatment of epithelial cells with the adhesin

Pre-treatment of human cells with the 61 kDa protein obtained by affinity adsorption with synthetic Synsorb H type 2, Le^a or Le^b showed inhibition of binding of *H. pylori* NCTC 11637; and, the ability to inhibit binding reflected the protein concentration in the eluates from the three different Synsorb blood group antigen preparations. The percentage inhibition was greatest for the protein obtained from H type 2 followed by that eluted from Le^b. The least inhibition was observed with the protein obtained from Le^a which was most probably related to the amount of protein eluted from the different Synsorb antigens.

Immunoelectron microscopic studies indicated that a 61 kDa heat shock protein is nearly exclusively associated with the outer surface of the OMP [Eschweiler *et al.*, 1993]. The *H. pylori* urease lacks a leader sequence and it was suggested that close association with the 61 kDa protein might support the transportation, stabilisation and folding of the urease [Eschweiler *et al.*, 1993]. Monoclonal antibodies raised against 60 kDa (61 kDa) heat shock protein effectively inhibited binding of the bacteria to cells [Hamid-Reza *et al.*, 1996].

Fauchere and Blaser [1990] have described loosely associated surface material of *H. pylori* that contain both urease and adherence activities. This adhesin bound to Hela cells and was not affected by neuraminidase treatment ; selective removal of sialic acid or preincubation of the bacteria with sialylated glycoprotein did not reduce

bacterial binding [Fauchere and Blaser, 1990]. Other work suggested that the *H. pylori* receptor on the gastric surface mucosa cells contains fucose [Falk *et al.*, 1993]. Borén *et al.* [1993] found no strong evidence for the involvement of blood group antigens other than Le^b in binding of *H. pylori* in their model system. They used tissue sections and assessed binding microscopically, but this study examined cells in suspension and detected binding by flow cytometry and different monoclonal antibodies.

5.4.5 Conclusion

The results in this chapter demonstrated direct binding of H type 2 and Lewis antigens to whole bacterial cells, OMP or the protein isolated by affinity purification. The results complement those obtained in chapter 4 which indicated that monoclonal antibodies to H type 2, Le^b and Le^a inhibited bacterial binding. The intensity noted with the dot blot assay paralleled the results of the microtitre assays for NCTC 11637 which was H type 2 > Le^b > Le^a. These results also reflected the amounts of the 61 kDa protein obtained by affinity purification with synthetic H type 2, Le^b and Le^a. If binding of oligosaccharides *in vitro* reflect their interactions with the bacteria *in vivo*, the greater binding of Le^b compared with Le^a might more effectively block some of the bacterial adhesin among secretors, thereby reducing density of colonisation.

Chapter 6

The inflammatory responses to *H. pylori* in relation to ABO blood groups and disease

6.1 Introduction

While epidemiological studies indicated that individuals of blood group O or non-secretors are over represented among patients with peptic ulcers, among those with gastric carcinoma there are significantly more of blood group A [summarised by Mourant *et al.*, 1978]. These finding indicate factors other than presence of the bacteria, perhaps the host immune or inflammatory responses contributes to development of disease associated with *H. pylori* infection.

Epidemiological studies suggest chronic infection with *H. pylori* is associated with ischaemic heart disease (IHD) [Mendall *et al.*, 1994; Glynn, 1994; Miragliotta *et al.*, 1994; Martin-de-Argila *et al.*, 1995]. Patel and colleagues reported that *H. pylori* infection might contribute to increased risk of IHD by increasing fibrinogen levels among patients with *H. pylori* infection [Patel *et al.*, 1994] as these bacteria produce a tissue factor with procoagulant like activity which can convert fibrinogen to fibrin [Miragliotta *et al.*, 1989]. Plasma fibrinogen is an independent risk factor for IHD and cardiovascular disease [Kannel *et al.*, 1987; Qizibash *et al.*, 1991]. The increase of plasma fibrinogen produced by hepatocytes is mediated mainly by interleukin-6 (IL-6) produced by monocytes and macrophages [Akira and Kishimoto, 1992]

In most persons infected with *H. pylori* there is an increase in chronic inflammatory cells in the lamina propria including lymphocytes, monocytes, eosinophils and plasma cells [Blaser, 1992]. Cytokine proteins produced by various cell types are important mediators of inflammatory responses. Local increases in IL-6 have been associated with bacterial infections both at mucosal [De Man *et al.*, 1989] and non-mucosal sites [Helfgott *et al.*, 1989; Bhardwaj *et al.*, 1989]. The supernatants of gastric mucosal biopsy specimens from patients with *H. pylori* gastritis had high levels of tumour necrosis factor alpha (TNF) and interleukin 6 (IL-6) [Crabtree *et al.*, 1991].

Chronic infection and inflammation have been recognised as risk factors for a variety of human cancers and it has been proposed that active oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical generated in inflammed tissues can cause injury to target cells and also damage DNA. These responses could contribute to tumour development [reviewed by Ohshima and Barsch, 1994]. There is now increasing evidence to suggest that nitric oxide (NO) and its derivatives produced by activated phagocytes might also play a role in the multistage carcinogenesis process [reviewed by Ohshima and Barsch, 1994]. Nitric oxide also inhibits platelet and leukocyte adhesion to endothelial cells as well as mediating platelet plug disaggregation [Radomski *et al.*, 1987; Moncada *et al.*, 1988; Gryglewski *et al.*, 1988; Bath *et al.*, 1991]

The aims of this part of the study were: 1) to determine if there was evidence for *H. pylori* infection among IHD deaths in the local population and 2) to compare inflammatory responses (IL-6, TNF or NO) from human buffy coats of blood group O and group A exposed to *H. pylori* antigens.

6.2 Material and methods

6.2.1 Antigen preparation

Antigen preparations for the *H. pylori* ELISA were basically performed according to the method of Newell (1986). *H. pylori* NCTC 11637 was cultured as described in chapter 2. Bacterial cells were harvested and washed twice in 5 ml of distilled water and suspended in 0.2 M glycine hydrochloride buffer (pH 2.2) at a concentration of 0.1 g of cells to 2.5 ml of buffer. The suspension was gently shaken for 15 min at room temperature and then centrifuged at 11 000 x *g* for 15 min at 4°C. The supernatant was dialysed overnight against sterile distilled water at 4°C.

6.2.2 Detection of antibodies to *H. pylori* in autopsies

Batches of 96 well microtitre plates were coated with 100 µl of 5µg ml^{-1} of *H. pylori* NCTC 11637 antigens and incubated overnight at 4°C as described in 3.2.6. The plates were washed with washing buffer and blocked with blocking buffer. Serum from autopsy samples diluted 1/100 in blocking buffer were added to the wells for detection of IgG and incubated at 37°C for 60 min then washed with washing buffer. HRP-anti-human IgG (Sigma) diluted 1/200 in blocking buffer were added to the plates and incubated for 60 min at room temperature. The assay was continued in the same way as the whole cell ELISA for determination of antibodies in patients serum (3.2.7) . The assays for all autopsy samples were examined at the same time under the same conditions for specific IgG.

6.2.3 Collection of human peripheral blood monocytes

Buffy coats (50 ml) were obtained from the Scottish National Blood Transfusion Service and diluted 1: 2 using sterile PBS under aseptic condition. Diluted blood (15 ml) was layered carefully on histopaque (5 ml) (Sigma, Poole, UK) in sterile plastic

centrifuge tubes and centrifuged for 30 min at 300 x *g*. Mononuclear leukocytes were collected from the interface, and washed twice with pyrogen free PBS or DMEM (Gibco) then resuspended in DMEM with 10% human serum, each individual buffy coat with it's respective serum.

6.2.4 Determination of ABO blood groups

The ABO blood group of the donors was determined from blood specimens by slide agglutination with monoclonal anti-A and anti-B antibodies (2.3)

6.2.5 Stimulation of human monocytes

Buffy coats were resuspended in DMEM with 10% human serum with streptomicin (200 μg ml^{-1}) and penicillin (100 IU ml^{-1}) and adjusted to 2 x 10^6 ml^{-1}. Cells (500 μl/well) were placed in 24 well tissue culture plates. They were then mixed with 500 μl of different concentrations of whole bacteria, OMP or purified adhesin of *H. pylori* NCTC 11637 suspended in DMEM containing 10% FCS without antibiotics were added to the plates. Controls contained only medium with 10% FCS and cells. The plates were incubated in a 37°C in a humidified 5% CO_2 incubator. At different intervals, the supernatants were collected in sterile tubes, centrifuged at 300 x *g* for 10 min, collected and used directly to estimate the amount of TNF, IL-6 or NO.

6.2.6 ELISA for detection of IL-6

IL-6 levels were measured with a solid phase ELISA. Microtiter flat-bottomed plates (96 wells) were coated with 100 μl/well of 0.5 μg ml^{-1} mouse monoclonal antibody specific for IL-6 (R&D Systems, Abingdon, UK) in coating buffer (2.1.1) and incubated over night at 4°C. They were washed 6 times with washing buffer (2.1.3) and 100 μl/well of blocking buffer (2.1.4) added and incubated for 30 min then removed. Supernatant samples (100 μl) were added to duplicate wells.

Dilutions of recombinant human IL-6 standard (R&D) in blocking buffer were used as positive controls. The plates were incubated for 2 hr at 37°C with continuous shaking. The plates were washed 6 times and 100 µl of goat polyclonal anti-human IL-6 (R&D) was added to the wells and incubated for 2 hr at 37°C with continuous shaking. After the plates were washed 6 times, 100 µl of HRP conjugated donkey anti-goat IgG were added to the wells for 1 hr at 37°C with continuous shaking then washed 6 times with washing buffer. The substrate (100 µl) (2.1.6) was activated by 40 ml H_2O_2 (30% v/v) immediately before use and added to the wells. The colour change was stopped after 10 - 20 min by adding 100 µl of H_2SO_4 (2.1.7). The optical density at 490 nm was determined by an ELISA plate reader (Dynatech) and corrected by subtracting the OD of the corresponding blank well containing each of the components except the supernatant from the cells. The amount of IL-6 in each sample was determined relative to the human IL-6 standard curve and results were expressed in ng ml^{-1}.

6.2.7 TNF bioassay

The L929 mouse fibroblast cell line was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK) and cultured in growth medium DMEM containing 10% FCS. L929 cells were dislodged by 0.1% trypsin (w/v) (Gibco, Paisley, UK) to avoid cell clumping and washed with DMEM by centrifugation at 300 x *g*. They were resuspended in growth medium and adjusted to 3.0 x 10^5 ml^{-1}. Cells (100 µl) were placed in flat-bottomed 96 well tissue culture plates and incubated at 37°C in a humidified incubator containing 5% CO_2 for 24 hr. The growth medium was discarded and replaced with 100 µl / well of test supernatants diluted 1: 1 in assay medium (DMEM containing 5% FCS, 1 mM L-glutamine and 2 µg ml^{-1} actinomycin D (Sigma, Poole, Dorset). The supernatants were tested in triplicate. Series of two fold dilutions of a standard of recombinant human TNF (NIBSC, Hertfordshire, UK) in assay medium containing 1 µg ml^{-1}

actinomycin D. The concentrations which ranged from 2 to 1000 IU ml^{-1} were added to wells in triplicate. TNF standard was used in every plate. In each assay, 6 wells with 100 µl of assay medium containing 1 µg mL^{-1} actinomycin D were included as controls and another 6 wells without L929 cells were included in each plate as blanks. All were treated identically to the test samples. Plates were then incubated at 37°C in a humidified incubator containing 5% CO_2 for another 24 hr. The supernatant in each well was removed and replaced with 50 µl crystal violet (0.5% w/v in 20% methanol in distilled water which had been filtered through Whatman no 1 filter paper. After 2 min, plates were washed with tap water and allowed to dry. Each well received 50 µl 20% acetic acid to solubilise the stained cells. Any air bubbles were removed and optical density was measured at 570 nm with a microplate reader (MR 700 Dynatech Laboratories). The amount of TNF in each sample was determined relative to the TNF standard curve and results were expressed in IU ml^{-1}.

6.2.8 Nitric oxide assay

Nitric oxide production was assessed by measuring nitrite, a stable metabolic product of NO, in supernatants of the mononuclear phagocytes [Zhang *et al.*, 1994]. Griess reagent a 1:1 (v/v) mixture of 0.3% *N*-1 naphthyl ethylenediamine dihydrochloride (Sigma) and 1% sulfanilamide (Sigma) in 5% H_2PO_4 was added to 96 well tissue culture plates at 100 µl/well. Equal volumes of culture supernatants were added to the Griess reagent and incubated for 10 min at room temperature. The absorbence was determined on a MR 700 microplate reader (Dynatech Laboratories) at 570 nm. Results were compared to a standard curve of dilutions of sodium nitrate prepared for each experiment.

6.3 Results

6.3.1 Levels of IgG antibodies to *H. pylori* in autopsy specimens

Among 70 patients with no history of gastritis, peptic ulcer or gastric cancer, there were 37 patients whose primary cause of death was IHD. Diagnosis of IHD was made on the basis of evidence of narrowing or occlusion of one or more of the coronary arteries due to atheroma and/or thrombosis. In addition there could also be evidence of previous ischaemic damage to heart muscle, usually in the form of fibrosis. Serum samples from 33 age and sex matched individuals whose recorded cause of death was accidental were used as controls.

All autopsy serum samples were diluted 1/100 and screened for specific IgG for *H. pylori*. There were higher IgG levels specific for *H. pylori* in sera from IHD deaths (mean OD = 1.001) compared with those obtained with sera from accidental deaths (mean OD = 0.770) ($p < 0.06$). Detailed examination of the autopsy findings revealed that 8 of the accident victims had significant evidence of heart disease, although this was not the primary cause of death. Re-analysis of the results in which the accident victims with evidence of heart disease were included in the IHD group found significantly higher levels of IgG to *H. pylori* among the IHD group (mean = 1.010) compared with the accident group (mean = 0.690)($p < 0.009$) (Table 6.1)

Assessment of the data by age found that the levels of IgG to *H. pylori* were higher among the IHD group who died earlier (the 40 - 59 years age range). This difference was not observed among samples from the two groups in the age range > 60 years (Figure 6.1).

There were no significant differences observed for specific IgG levels for *H. pylori* in autopsy serum samples from IHD cases compared with accident cases with references to blood group (Table 6.1).

6.3.2 The effect of incubation time on release of TNF, IL-6 and nitric oxide from human buffy coat

The IL-6 and TNF production was examined by stimulating human monocytes (2 x 10^6) with OMP of *H. pylori* NCTC 11637 at a concentration of 1.9 μg ml^{-1} at 4, 8, 18, 42, 66 and 90 hr. The results showed that TNF was released at 4 hr after stimulation and the amount detected increased progressively until 18 hr when it started to decrease. IL-6 was detected at 4 hr after stimulation but increased progressively until 42 hr when it started to decrease. Nitric oxide was released 42 hr after stimulation with the highest OD obtained at 66 hours (figures 6.2, 6.3 and 6.4)

6.3.3 IL-6 released in response to *H. pylori* antigens

6.3.3.1 The effect of purified adhesin and OMP on release of IL-6

IL-6 release was assessed for different concentrations of whole cells, purified adhesin and OMP. The results were dose dependent. Release of IL-6 stimulated with purified adhesin or OMP at concentrations of 1.87, 3.75, 7.5 and 15 μg ml^{-1} was increased with increasing concentrations of antigen. Higher levels of IL-6 were obtained with purified adhesin compared with equivalent amounts of OMP with cells from 3 different donors (paired two-tail t-test). There was a significant release of IL-6 in response to adhesin compared with OMP ($P<0.03$) (Figure 6.5).

Table 6.1 Comparison of optical density of specific IgG for *H. pylori* with reference to blood group and cause of death, **A**) before and **B**) after accident individuals were screened for signs of heart disease (SE = standard error) P value assessed by Mann Whitney U test

	ACCIDENT n = 33		**IHD n = 37**		
A)	**mean**	**SE**	**mean**	**SE**	**P value**
Group A (n = 43)	0.75	0.11	0.97	0.11	P<0.1
Group O (n = 27)	1.001	0.16	1.04	0.12	P<0.8
All cases (n = 70)	0.770	0.08	1.001	0.08	P<0.06

	ACCIDENT n = 25		**IHD n = 45**		
B)	**mean**	**SE**	**mean**	**SE**	**P value**
Group A (n = 43)	0.72	0.12	0.97	0.11	P<0.1
Group O (n = 27)	0.900	0.23	1.06	0.10	P<0.3
All cases (n = 70)	0.690	0.09	1.010	0.07	P<0.009

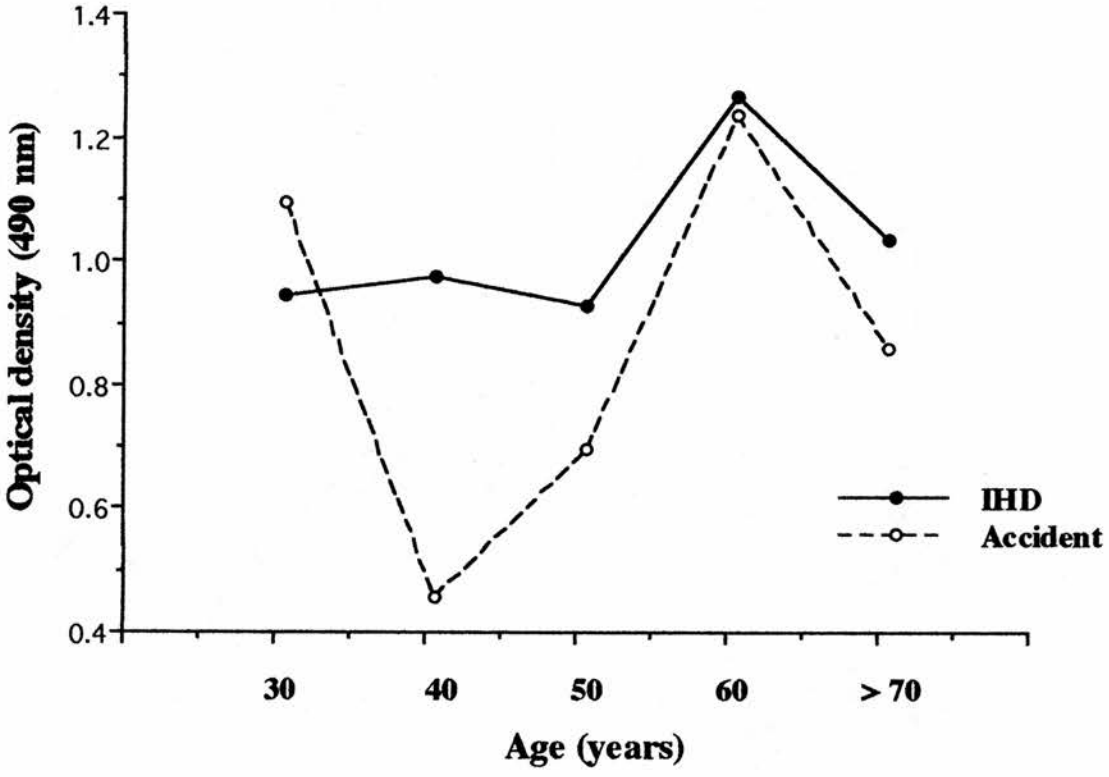


Figure 6.1. Levels of IgG to *H. pylori* assessed by age and evidence of IHD

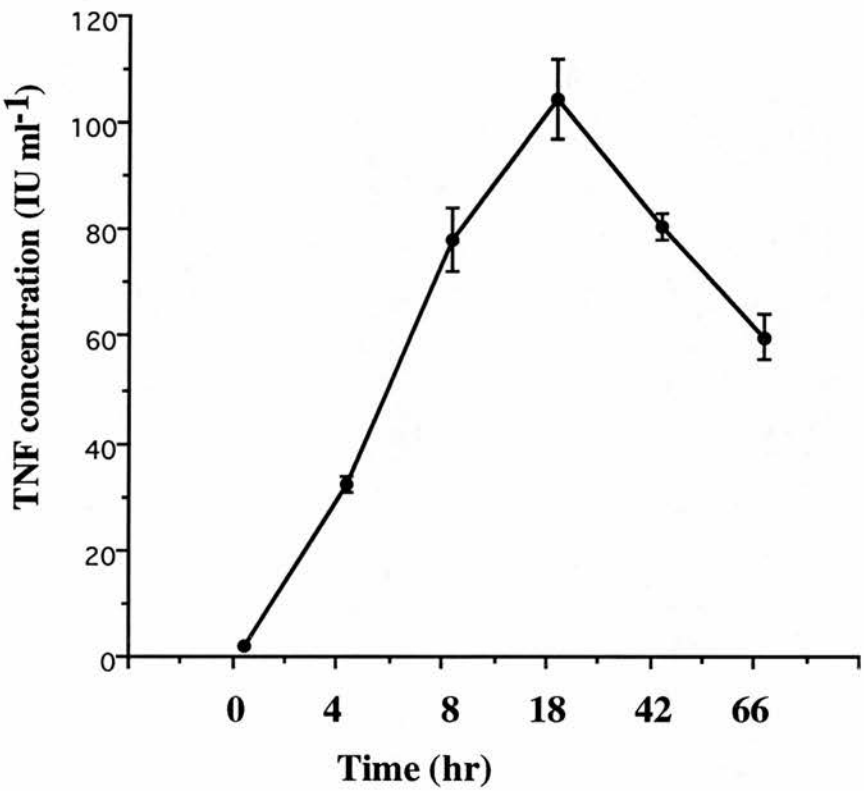


Figure 6.2 Mean of two experiments to assess the effect of incubation time on release TNF from buffy coats stimulated with OMP (1.9 μg ml^{-1}) of *H. pylori* NCTC 11637

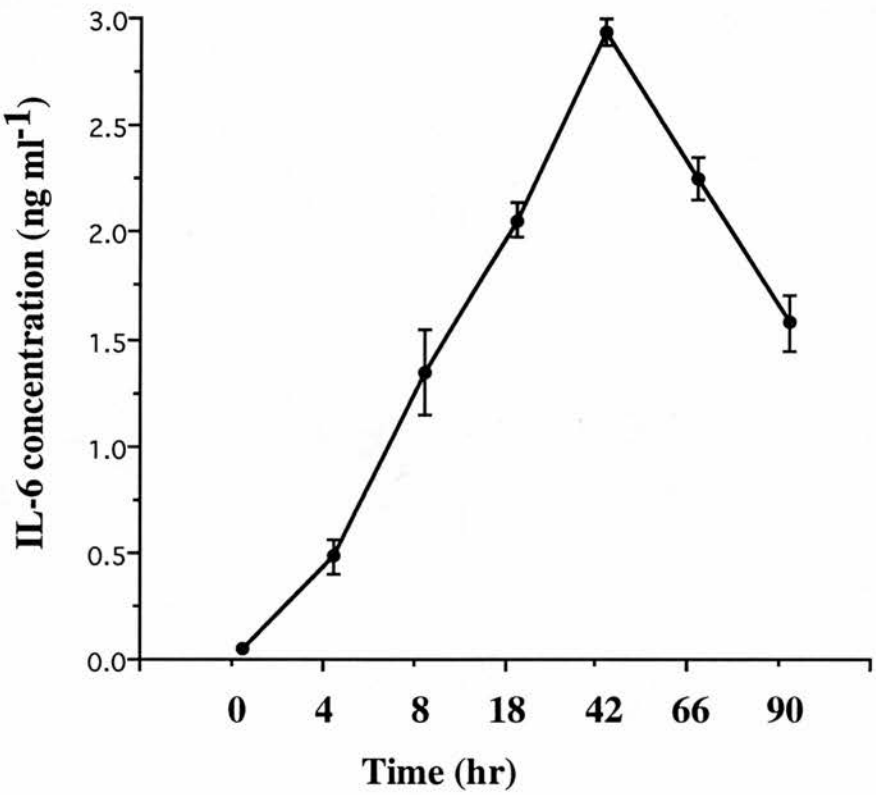


Figure 6.3 Mean of two experiments to assess the effect of incubation time on release IL-6 from buffy coats stimulated with OMP (1.9 μg ml^{-1}) of *H. pylori* NCTC 11637

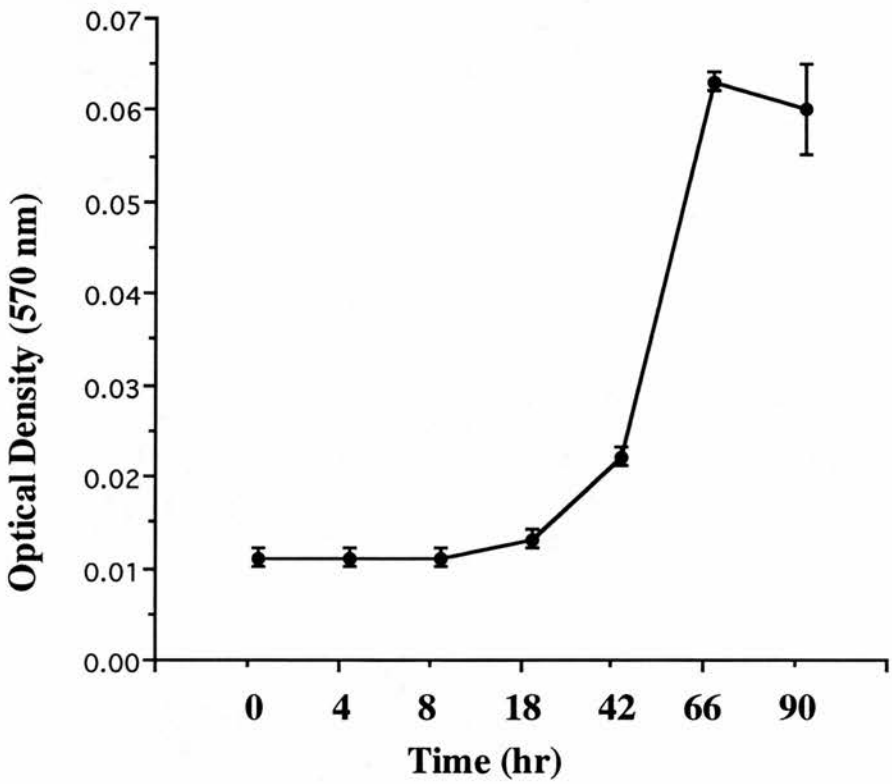


Figure 6.4 Mean of two experiments to assess the effect of incubation time on release nitric oxide from buffy coats stimulated with OMP (1.9 μg ml^{-1}) of *H. pylori* NCTC 11637

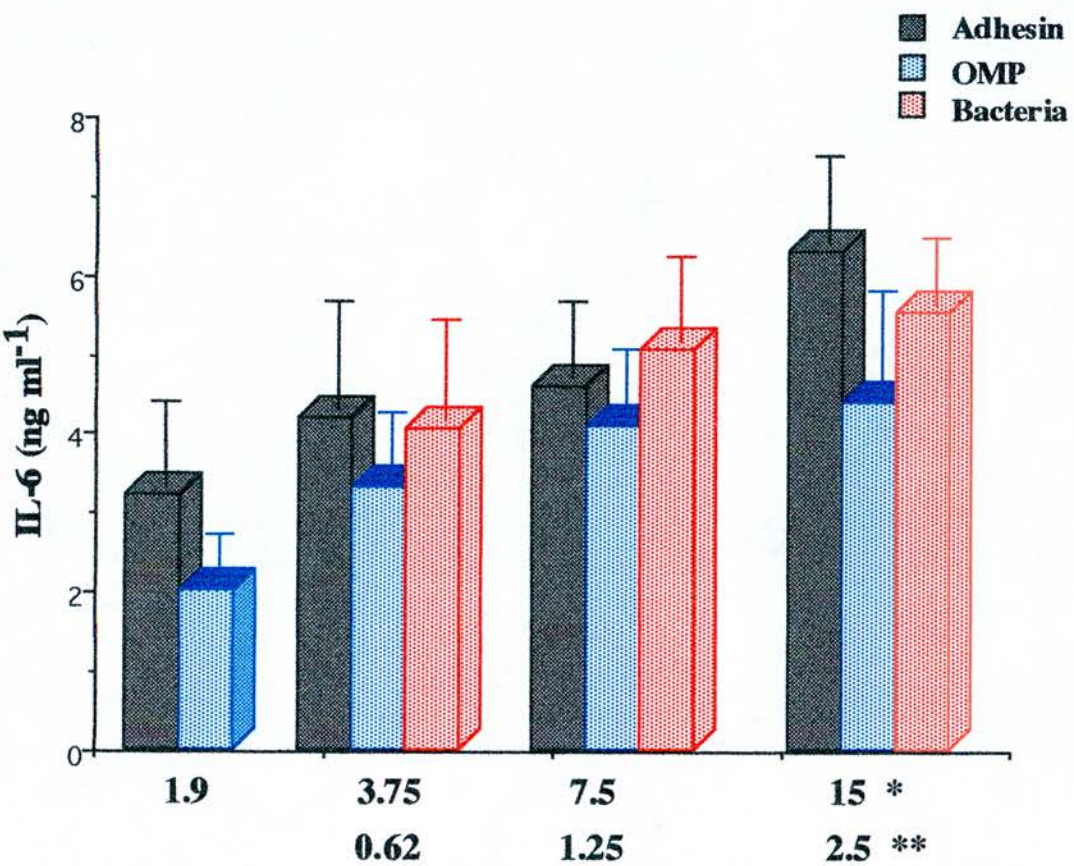


Figure 6.5 Mean of IL-6 released from human buffy coats of 3 individuals stimulated for 42 hours with different concentrations of whole cell, OMP or purified adhesin of *H. pylori* NCTC 11637.

* proteins μg ml^{-1} ** bacteria x 10^8 ml^{-1}

6.3.2.2 The effect of blood group on release of IL-6

Human buffy coats of 16 different individuals released IL-6 in response to stimulation with 1.9 μg/ml of purified adhesin. Those from individuals of blood group O (n = 8) had significantly higher IL-6 levels (mean 4.69 ng ml^{-1}) compared with IL-6 released from buffy coats obtained from blood group A individuals (n = 8) (mean 2.97 ng ml^{-1}) (Mann-Whitney U test, $P< 0.05$) (Figure 6.6).

6.3.3. TNF released in response *H. pylori* antigens

6.3.3.1 The effect of purified adhesin and OMP on release of TNF

TNF release in response to different concentrations of whole cell, purified adhesin and OMP was examined with buffy coats from 3 different individuals. At 8 hr following stimulation, the release of TNF from buffy coats in response to increasing concentrations of the purified adhesin or OMP was dose dependant. By the paired two-tail t-test, there was significantly increased release of TNF from the cells in response to adhesin compared with OMP ($P<0.003$) (figure 6.7).

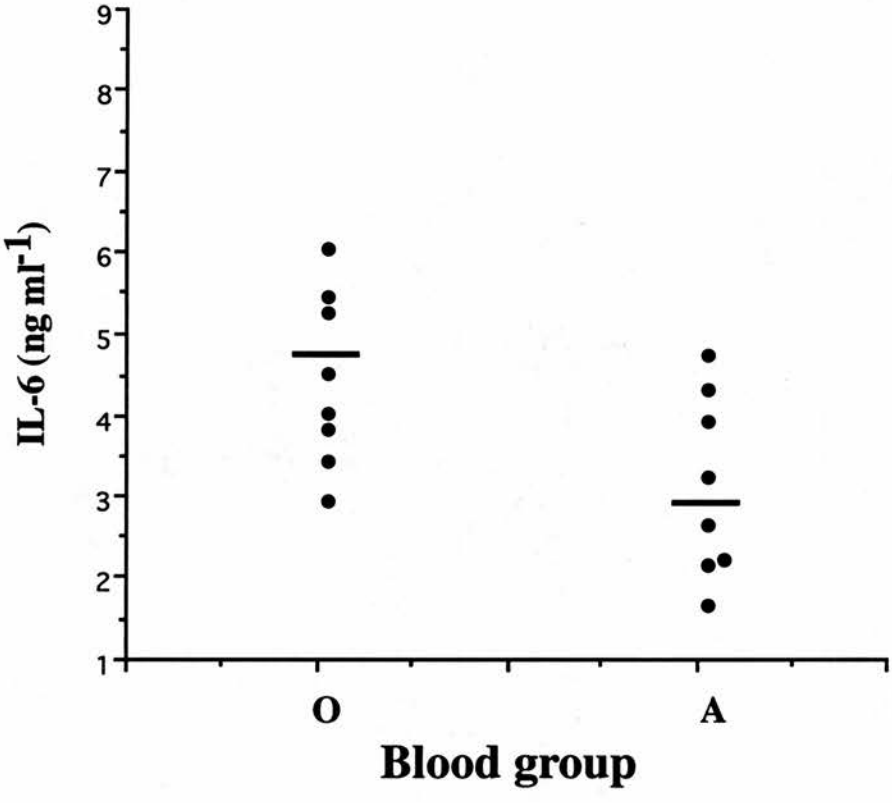


Figure 6.6 IL-6 released from cells of 8 group O and 8 blood group A donors stimulated with 1.9 μg/ml of purified adhesin after 48 hours.

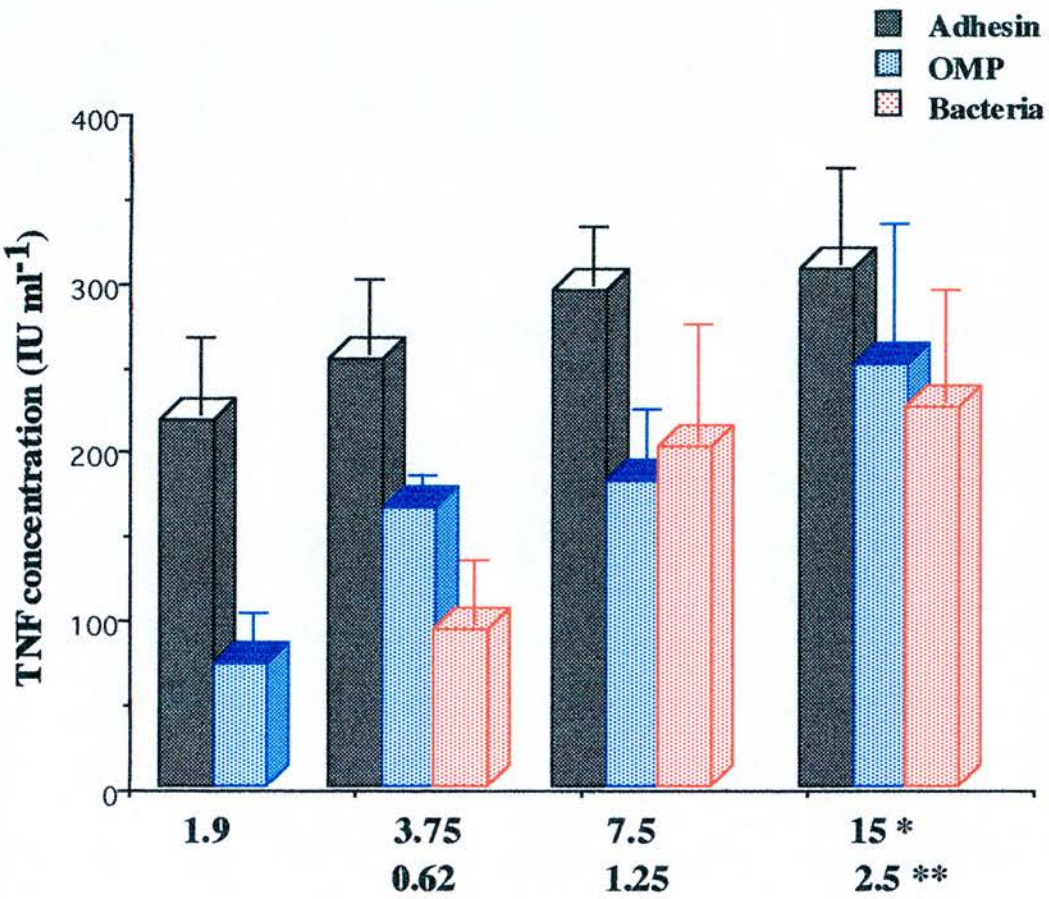


Figure 6.7 Mean TNF released from buffy coats of 3 different individuals stimulated for 8 hours with different concentration of whole cell, OMP or purified adhesin of *H. pylori* NCTC 11637.

* proteins μg ml^{-1} ** bacteria x 10^8 ml^{-1}

6.3.3.2 The effect of blood group on release of TNF

TNF released from human buffy coats of blood group O (n = 8) and blood group A (n = 8) donors in response to purified adhesin was assessed. The results showed that the mean TNF released from cells of blood group O individuals (262 IU ml^{-1}) was significantly higher than that from cells of blood group A individuals (190 IU ml^{-1}) (Mann-Whitney U test, $P<0.05$) (Fig 6.8).

6.3.4. Nitric oxide release in response to *H. pylori* antigens

6.3.4.1 The effect of OMP, purified adhesin and whole cell bacteria on release of nitric oxide

The assay detected nitrite at levels ranging between 1 to 100 μ mol^{-1}. Because some of the OD values in the experiments were below the lower limits of the standard, results are expressed as OD rather then μ mol^{-1}. Nitric oxide released in response to different concentrations of purified adhesin, OMP (1.25, 2.5, 5 and 10 μg ml^{-1}) or whole bacteria (0.625, 1.25 and 2.5 x 10^8 bacteria ml^{-1}) was assessed. In experiments with cells from 4 different donors, there was a dose dependent effect with **decreased** nitric oxide levels detected at 66 hr with **increasing** concentrations of the purified adhesin, OMP or whole bacteria. The levels of nitric oxide were higher for cells exposed to concentrations of whole bacteria compared with cells exposed to purified adhesin or OMP (Figure 6.9). In another experiment, cells from 3 different individuals were stimulated with a broader range of concentrations of adhesin. A similar pattern of nitric oxide levels was observed in the range of concentrations tested previously, but there was a biphasic pattern observed with the broader range of concentrations tested (figure 6.10).

6.3.4.2 The effect of blood group on release of nitric oxide

There were significantly higher levels of nitric oxide released from buffy coats of blood group O individuals (n = 8) stimulated with whole bacteria at 2.5 x 10^8 bacteria ml^{-1} for an incubation time of 66 hr compared with blood group A (n = 8) individuals . The mean optical density for blood group O individuals was (0.105) and blood group A (0.068) (Mann-Whitney U test, $P< 0.03$) (figure 6.11).

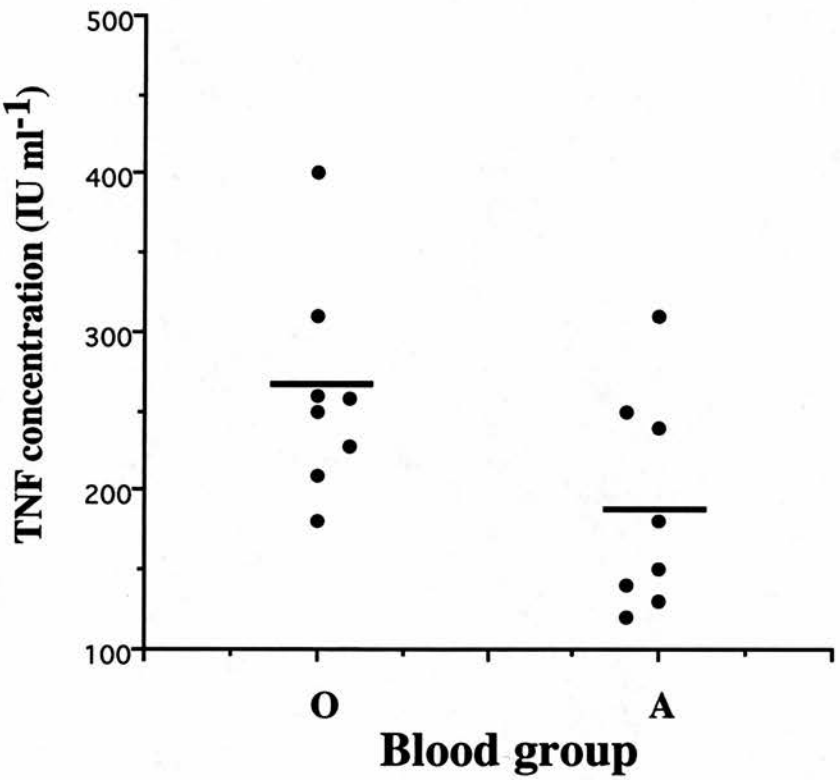


Figure 6.8 TNF released from 8 blood group O and 8 blood group A buffy coats stimulated with 1.9 μg/ml of purified adhesin after 8 hours incubation time.

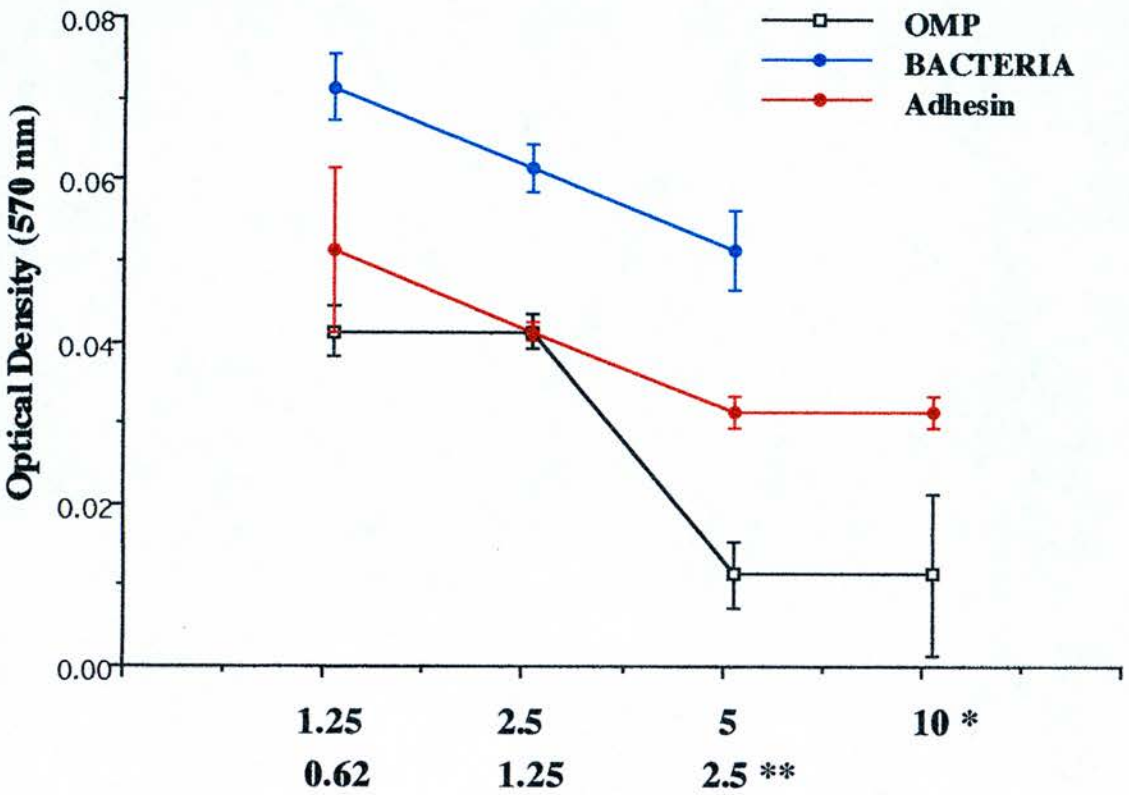


Figure 6.9 Mean nitric oxide released from buffy coats of 4 individuals stimulated for 66 hours with different concentrations of purified adhesin , OMP or whole cells of *H. pylori* NCTC 11637.

* protein µg ml^{-1} ** bacteria x 10^8 ml^{-1}

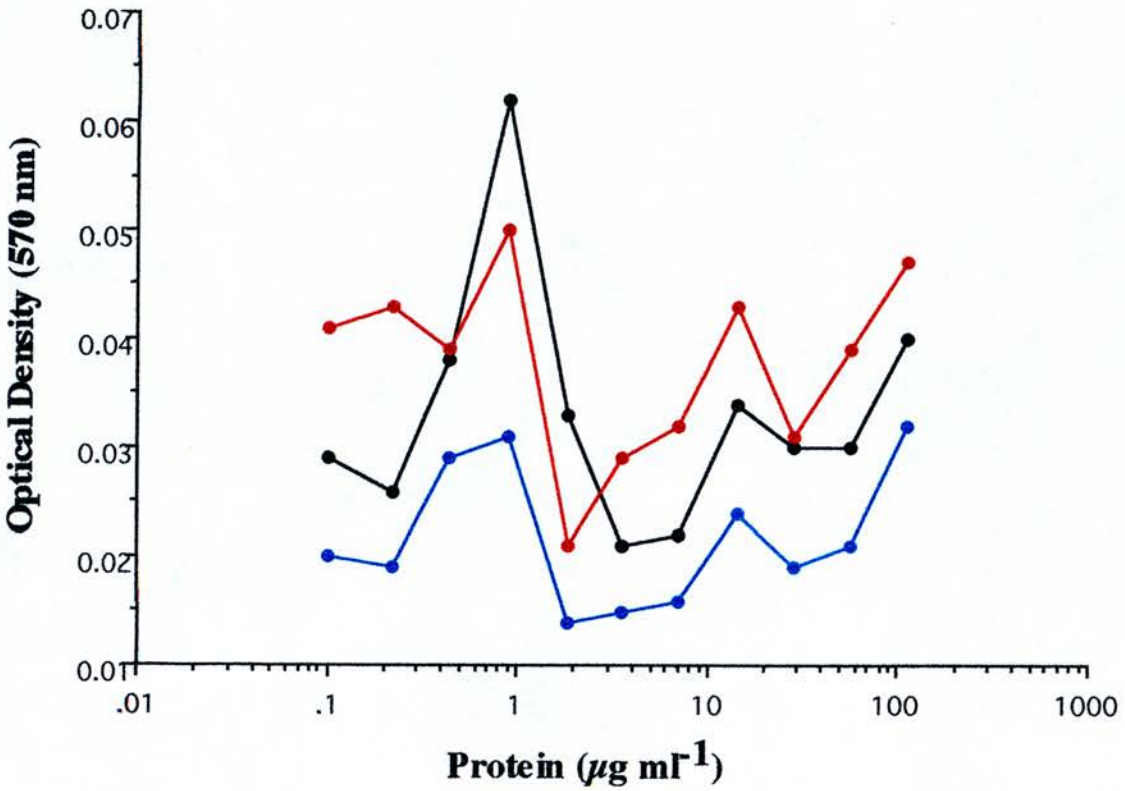


Figure 6.10 Nitric oxide released from buffy coats of 3 donors stimulated for 66 hours with different concentrations of purified adhesin of *H. pylori* NCTC 11637

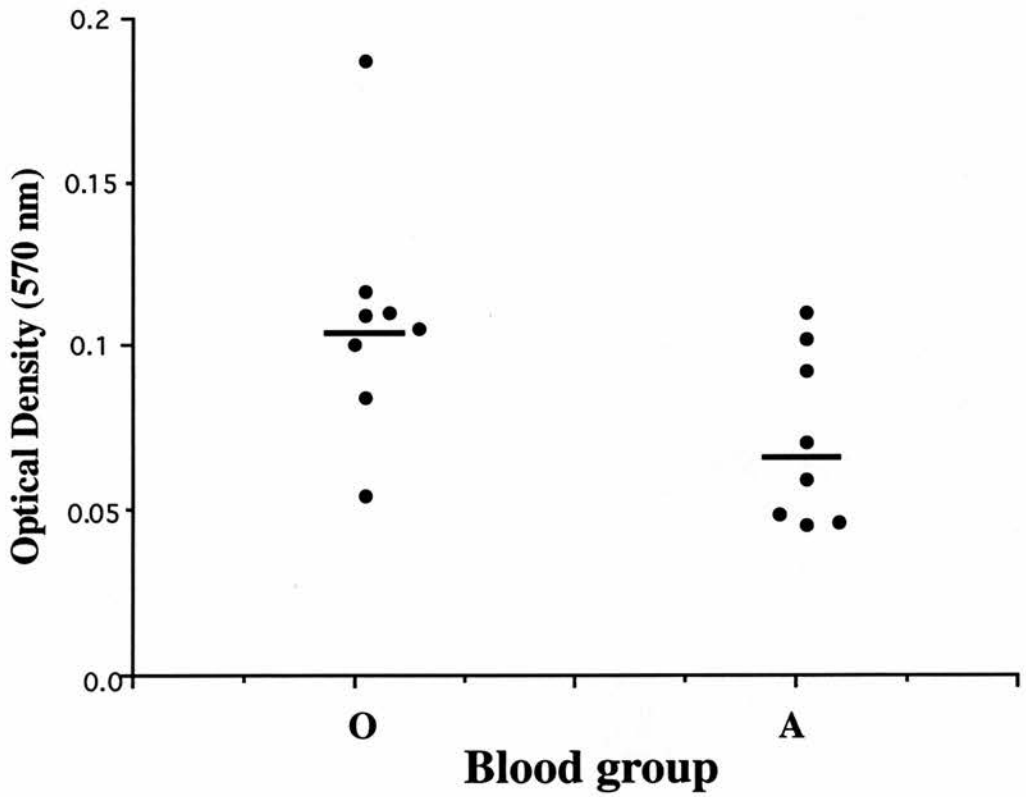


Figure 6.11 Nitric oxide released from 8 blood group O and 8 blood group A buffy coats stimulated with whole *H. pylori* NCTC 11637 (2.5 x 10^8 ml^{-1}) for 66 hours.

6.4 Discussion

The results are discussed with references to the objectives set out in the introduction.

6.4.1 IgG antibodies to *H. pylori* in relation to IHD

Most serological studies on *H. pylori* have been qualitative rather than quantitative. The level of IgG antibodies to *H. pylori* correlated with the density of antral colonisation by the bacteria and also with the degree of gastritis of the antrum [Kreuning *et al.*, 1994]. The results obtained with sera from IHD deaths and accident victims are in agreement with epidemiological studies which suggested that chronic infection with *Helicobacter pylori* is associated with coronary heart disease (IHD) [Mendall *et al.*, 1994; Glynn, 1994; Miragliotta *et al.*, 1994; Martin-de-Argila *et al.*, 1995]. In a case control study, Mendall and colleagues found that seropositivity to *H. pylori* conferred a twofold risk of coronary heart disease [Mendall *et al.*, 1994]. Morgando and colleagues observed an increase in the risk of myocardial infarction at younger ages for patients with *H. pylori* infection as judged by presence of antibodies in the blood [Morgando *et al.*, 1995]. Although the differences in levels of IgG for the younger age groups (40 - 59 years) were not significant, this supported the finding by Morgando *et al.*, [1995].

The advantage of using autopsy specimens is that evidence of asymptomatic heart disease can be taken into consideration in the analysis of the results. The cases of IHD examined were unexpected deaths which might indicates these were more severe attacks which resulted in death. Epidemiological studies of IHD identified an association between blood group A and myocardial infraction and also with thrombosis [summarised by Mourant *et al.*, 1978]. The opposite was observed in this study, there were more blood group O individuals who died from of IHD compared with group A but this was not significant. A similar trend was observed in

the one study reported in which postmortem material was examined [reviewed by Mourant and Kopéc, 1978]. These studies need to be expanded to examine larger numbers of autopsy samples and to compare these results with age and sex matched individuals who survived their first heart attack.

H. pylori isolated from biopsy specimens can coagulate blood by stimulating mononuclear cells. Following bacterial stimulation, mononuclear leukocytes produce a tissue factor with procoagulant-like activity which, through the extrinsic pathway of blood coagulation, converts fibrinogen into fibrin. This is suggested to contribute to the pathogenesis of coronary heart disease [Miragliotta *et al.*, 1994]. The effect of *H. pylori* gastritis on markers of inflammation such as fibrinogen concentration, circulating leukocyte count, C reactive protein and sialic acid concentration can be mediated via certain cytokines including TNF and IL-6. Concentration of both these mediators are increased in the gastric mucosa of *H. pylori* -infected patients [Crabtree *et al.*, 1991].

6.4.2 Inflammatory responses of monocytes of blood group A and O donors

The proinflammatory cytokines, (TNF, IL-1, IL-6 and IL-8) are a class of endogenous proteins that exert important influences on immune, haematological and metabolic responses to injury [Fong *et al.*, 1990]. They are active at low concentrations and are produced by a variety of cells, including lymphocytes, monocytes and neutrophils [Arai *et al.*, 1990]. A previous study of cytokines released from human buffy coats stimulated by *H. pylori* porins, showed release of various cytokines obtained with different porin concentrations. These results suggested that the surface components of *H. pylori* were able to induce a series of inflammatory responses [Tufano *et al.*, 1994]. The purified adhesin that binds H type 2 and Lewis antigens (chapter 5) was able to stimulate human buffy coats to release TNF, IL-6 and NO. TNF and IL-6 were detected at 4 h after the stimulus

and increased progressively until 18 h when TNF started to decrease. IL-6 levels increased progressively until 48 hr and then started to decrease. Detection of the cytokines was dose dependent with increasing TNF and IL-6 production observed with increasing concentration of whole bacteria, OMP and adhesin, suggesting that purified adhesin might play an important role in inflammatory responses in the pathogenesis of *H. pylori* infection as well as adherence.

Nitric oxide has been implicated in a number of physiological and pathophysiological functions in the gut. Decrease in gastric mucosal blood flow has been identified as a prerequisite to the development of acute erosions and stress ulcers [Mersereau and Hinchey, 1973]. Nitric oxide synthesis has been detected in the gastric mucosa and appears to play a role in protecting it during physiological stress by acting as an endogenous vasodilator supporting mucosal blood flow [Stark and Szurszewski, 1992; Whittle *et al.*, 1992].

6.4.3 Factors contributing to gastroduodenal disease

Development of more serious gastroduodenal disease might be related to infection with ulcerogenic or carcinogenic strains of *H. pylori* [Blaser, 1994; Xiang *et al.*, 1995]. An increased prevalence of antibody against *Cag A* has been reported in both peptic ulcers disease and gastric cancer patients [Tummuru *et al.*, 1993; Crabtree *et al.*, 1993]. Studies using polymerase chain reaction amplification and DNA hybridization to probe for the presence of *CagA* in clinical isolates were not significantly associated with duodenal disease [Owen *et al.*, 1994]. Hazell and colleagues in a study of an Australian population reported the prevalence of antibodies against *CagA* in the duodenal ulcer group was higher than that found in the control group; however, in a Chinese population, no significant difference was found between the prevalence of antibodies to the *CagA* antigen in asymptomatic subjects compared with gastric cancer patients [Hazell *et al.*, 1996]. Analysis of the

antibody responses to other *H. pylori* antigens such as the 30 kDa and 45 kDa antigens identified an association with gastric cancer [Hazell *et al.*, 1996] and cluster analysis of the Western blot profiles showed that sera from gastric cancer patients reacted with almost all the major antigens of *H. pylori* tested [Hazell *et al.*, 1996].

Chronic infection and inflammation have long been recognised as risk factors for a variety of human cancers. Nitric oxide and other oxygen radicals produced in infected and inflamed tissues could contribute to the process of carcinogenesis [reviewed by Ohshima and Bartsch, 1994].

There were no published papers on the production of nitric oxide from human buffy coats in response to *H. pylori* ; therefore, part of this study was designed to examine the effect of whole cells of *H. pylori* , OMP and purified adhesin on the ability of human buffy coats to release nitric oxide. The results showed that whole cells, OMP or purified adhesin induced nitric oxide and stimulation of human buffy coats with whole cell bacteria produced higher amount of nitric oxide compared with purified adhesin or OMP. If nitric oxide plays a role in gastric cancer, these results suggested that the whole bacteria are the important factor for releasing higher levels of nitric oxide. In contrast to the results obtained for TNF and IL-6, the lower concentrations of whole bacteria released higher levels of nitric oxide. If the lower levels of binding of *H. pylori* to epithelial cells of donors of group A compared with group O (chapter 4) reflects the circumstances *in vivo*, the association between *H. pylori* infection and gastric cancer might be related to lower density of colonisation.

6.4.4 Factors contributing to IHD

Decreased levels of nitric oxide have been implicated as a mechanism of primary or essential hypertension [Snyder and Bredt, 1992; Winquist *et al.*, 1984]. The inability of vessels to vasodilate appears to be related to decreased nitric oxide release and not to the reduced ability of smooth muscle cells to respond to nitric oxide [Shimokawa

and Vanhoutte, 1989; Minor *et al.*, 1990]. The degree of endothelial dysfunction parallels the degree of hypertension. The inability of vessels to vasodilate appears to be related to decreased nitric oxide release [Shimokawa and Vanhoutte, 1989; Minor *et al.*, 1990]. The hypothesis tested in this chapter was that cells of blood group A donors might release more IL-6, TNF or nitric oxide in response to *H. pylori* compared with blood group O; however blood group O individuals released significantly higher amount of IL-6, TNF and nitric oxide compared with blood group A in response to *H. pylori* and higher numbers of *H. pylori* results in lower levels of nitric oxide. These studies need to be repeated with endothelial cell to determine if these bacteria or their antigens have a similar effect on induction of nitric oxide from endothelium.

The association between blood group A and liability to IHD or disease involving clotting might be explained by the observation that people of blood group A have higher plasma levels of factor VIII (anti-haemophilic globulin) than do those of blood group O [reviewed by Mourant and Kopéc, 1971] and lower levels of nitric oxide observed with blood group A responses to *H. pylori* might results in lower levels of vasodilator and reduction in desolution of clot formation or platelet aggregation. The interactions between levels of *H. pylori* and blood group need to be assessed further in relation to the production of procoagulant activity reported by Miragliotta *et al.*, [1989].

Further studies will be required to determine if *H. pylori* infected patients have high levels of coagulation factors compared with *H. pylori* negative patients or if cells of group A individuals release higher levels of platelet activation factor in response to these bacteria.

Chapter 7

General discussion

This chapter summarises the findings and conclusions with reference to the original objectives to the study, highlights the applications or limitations of the experiments and methods of analysis used throughout this study and suggests future work.

7.1 Objectives of the study

Epidemiological studies carried out before *H. pylori* was identified found that non-secretors and individuals of blood group O were over-represented among patients with peptic ulcers. This study tested the mechanisms that might explain how these genetic susceptibility factors could aid the initial colonisation with *H. pylori.* The first objective was to assess genetic and life style factors associated with isolation of *H. pylori* from patients undergoing gastroscopy. The second objective was to study how the genetic and environmental factors associated with peptic ulcer disease might affect binding of *H. pylori* to epithelial cells. These included the effect of ABO, Lewis blood groups, smoking and fasting.

7.2 Isolation of *H. pylori* from gastroscopy patients

Skirrow's medium has been used for the isolation of *H. pylori* from gastric biopsies, but is not generally recommended because 14% of the isolates can be inhibited by nalidixic acid and polymyxin B can inhibit 5% of isolates [Dent and McNulty, 1988]. In this study, one (1/52) of the isolates was inhibited by polymyxin B and another was inhibited with colistin on primary isolation; therefore for the local isolates the use of Dent's medium with cefsulodin is recommended for primary isolation of *H. pylori* from gastric biopsies.

The results confirmed the diagnostic value of ELISA for determination of serum IgG antibodies against *H. pylori*. The results of the ELISA assay significantly correlated with the presence of *H. pylori* in gastric biopsies determined with CLO, microscopy and culture. In addition, the assay differentiated between patients with and without peptic ulcers. These results suggest that ELISA might be useful for screening patients refered for endoscopy thereby reducing the endoscopy list in the clinic. As *H. pylori* identification in gastric cancer patients is lower than studies in patients with peptic ulcers, culture, endoscopy and levels of serum antibodies among patients with gastric cancer must be carried out to determine how useful serology would be for screening.

H. pylori positive patients tended to be older than non-infected patients and prevalence of the infection increased with age. The peak of *H. pylori* positive patients was found in the group aged between 50 - 60 years old, whereas the isolation rate tended to be lower in older patients (> 60 years). This has been associated with progression of chronic *H. pylori* gastritis to atrophic gastritis in elderly people, because severe gastric atrophy seems to make the stomach inhospitable to *H. pylori* [Goldschmiedt *et al.*, 1991, Kaneko *et al.*, 1992].

The first objective in the clinical survey was to examine the prevalence of *H. pylori* infection in relation to blood groups and secretor status. The study failed to show an increased prevalence of *H. pylori* infection among non-secretor patients compared with secretors, but blood group O was over-represented among patients with gastroduodenal disease and among patients with *H. pylori* infection. Larger number of patients need to be examined as only 52 were infected.

Smokers are more at risk of gastroduodenal disease, but there was no overall correlation between *H. pylori* infection and smoking. A significant correlation between *H. pylori* infection and smoking was found when results were analysed by

years of smoking. Thus indicating that longer term smoking is probably a risk factor for *H. pylori* infection.

7.3 Detection of H type 2 and Lewis antigens proposed to act as receptors for *H. pylori* on the gastric mucosa and cells used in the studies.

Monoclonal antibodies against H type 2, Le^a or Le^b bound to buccal epithelial cells, gastric mucosa and the Kato III cell line. Previous studies of human gastric carcinoma and normal gastric tissue has demonstrated blood group antigens by immunohistochemical methods [Sakamoto *et al.*, 1989] and the present study confirmed binding of monoclonal antibodies to these blood group antigens on the cell surface of normal gastric tissue by flow cytometry techniques.

Buccal epithelial cells bound the three monoclonal antibodies and those from individuals of group O had significantly higher binding indices for anti-H type 2 when compared with binding indices for non-O donors. H type 2, the antigen of blood group O, is found on the cells of all individuals except the very rare Bombay phenotype. There were no significant differences in binding indices for anti-Lewis antibodies for secretors compared with non-secretors. This might be due to cross reactivity of the anti-Le^b antibody with Le^a and the highly variable expression of Le^a on cells of secretors [Saadi *et al.*, 1993]. There were no differences in binding indices for males compared with females.

Fasting significantly reduced binding to BECs of the monoclonal antibody to H type 2 but not binding of monoclonal antibodies against the Lewis antigens. Binding indices for all three monoclonal antibodies were significantly lower for cells from fasting smokers compared with non-smokers. For both smokers and non-smokers levels of Lewis antigens were higher when the donor were fasting but the differences were not significant. The significant reduction of anti-H type 2 binding to BEC of fasting individuals might be related to lower saliva output associated with fasting.

This suggestion is supported by observations of other workers in which deprival of salivary epidermal growth factor (EGF) significantly lowered levels of carbohydrate, lipids and covalently bound fatty acids, but resulted in higher protein levels when compared with normal saliva [Sarosiek *et al.*, 1988].

Although fasting and smoking affected binding of monoclonal antibodies, the results of the bacterial binding studies with BECs indicated that the binding indices for *H. pylori* were significantly associated with binding indices for monoclonal antibodies to H type 2 and Le^b under all the conditions tested. The terminal fucose structure present on these blood group antigens found in the body fluid of secretors such as the H type 1 structure found in $Lewis^b$, A-$Lewis^b$ or B-$Lewis^b$ might bind to the adhesin and reduce colonisation.

Smokers have been shown to have an enhanced adherence of *Streptococcus pneumonia* to BECs and *Haemophilis influenzae* to their pharyngeal cells [Mahajan and Panhotra, 1989; Raman *et al.*, 1983; Fainstein and Musher, 1979]. While smoking has been identified as a risk factor for ulcers, the studies of *H. pylori* binding to BECs from smokers and non-smokers suggested that smoking will not result in increased bacterial binding or expression of H type 2 or Lewis blood group antigens which are proposed to act as receptors if there are similar effects on epithelial cells lining the gastric mucosa.

7.4 Inhibition of binding of *H. pylori* with monoclonal against Le^a, Le^b and H type 2

The second objective was to determine if in addition to Le^b previously identified as a receptor for *H. pylori* [Borén *et al.*, 1993], Le^a or H type 2 could also act as receptors for the bacteria. There was evidence that receptors for *H. pylori* on gastric mucosa cells contained fucose [Falk *et al.*, 1993]. Borén *et al* [1993] found no strong evidence for the involvement of fucose-containing blood group antigens other

than Le^b in binding of *H. pylori* in their model system in which binding of the bacteria to tissue sections was assessed microscopically [Borén *et al.*, 1993]. In contrast, the results of this study indicate that pretreatment of cells with monoclonal antibodies to H type 2, $Lewis^a$ or $Lewis^b$ are capable of inhibiting *H. pylori* binding to Kato III and BEC cells in suspension detected by flow cytometry binding studies. The results suggested that H type 2, Le^a and Le^b might be receptors for *H. pylori* on the gastric mucosa and that H type 2 found on almost all individuals is a key receptor for *H. pylori*

7.5 Direct evidence for H type 2, Le^a and Le^b to act as receptors for *H. pylori*

Studies in which bacterial binding is inhibited by pretreatment of target cells with polyclonal or monoclonal antibodies to the putative receptor have been criticised in that the inhibition of bacterial binding might be due to stearic hindrance. The antibodies are binding an epitope near the receptor and blocking access of the bacteria.

Direct binding of biotinylated H type 2, Le^a and Le^b to whole bacteria, OMP and purified adhesin provided complementary evidence that these antigens act as receptors for *H. pylori.* The microtitre spectrophotometric method provided a rapid, quantitative and reliable assay for screening the clinical isolates to determine what proportion of bacteria bound the blood group antigens. The assay has proved sensitive, reproducible and was used to demonstrates binding of there antigens to all 51 isolates tested. The use of direct attachment of biotinylated blood group antigens to the OMP, purified adhesin and to the intact whole cell for *H. pylori* NCTC 11637 showed binding of H type 2 to be significantly greater than Le^b and Le^a. These results also correlated with the amount of the 61 kDa adhesin obtained by affinity purification from the three antigens.

H. pylori expresses multiple adhesins on the cell surface such as the 20 kDa, 25 kDa and 59 kDa haemagglutinin components [Evans *et al.*, 1993; Huang *et al.*, 1992]. The results suggested that the 61 kDa protein a stably expressed adhesin is a major adhesin by which *H. pylori* binds to epithelial cells. The majority of strains bound H type 2, Le^b and Le^a in a pattern similar to that of NCTC 11637.

7.6 ABO blood groups and susceptibility to diseases associated with *H. pylori* infection

7.6.1 Peptic ulcers

Many bacterial pathogens that infect various mucosal sites colonise these surfaces by adhering to specific receptors in the mucus layer and to glycoconjugates on epithelial cells [Wadstrom and Trust., 1984]. The ability of a pathogen to adhere to host structures is often pivotal in the subsequent establishment of infection . It has been demonstrated that density of colonisation is related to inflammatory responses, epithelial damage and duodenal ulcer among patients infected with *H. pylori* [Atherton *et al.*, 1996].

7.6.1.1 Blood group and density of colonisation

The increased susceptibility of group O individuals to peptic ulcer disease might be due to enhanced binding of *H. pylori* to the H type 2 expressed in higher levels on cells of group O. Both secretors and non-secretors express H type 2 on their cells and secretors express Le^b in mucus. If the terminal fucose structure present on H type 2 is a major receptor, antigens found in body fluids of secretors with the terminal fucose on the type 1 precursor chain (H type 1, $Lewis^b$, A-$Lewis^b$ or B-$Lewis^b$) might bind to the adhesin and reduce density of colonisation.

7.6.1.2 Blood group and inflammatory responses

Whole cells, OMP and the purified adhesin were able to stimulate human buffy coats to release TNF, IL-6 and NO. The results suggest that the adhesin might also play an important role in induction of inflammatory responses during *H. pylori* infection as well as adherence to the epithelial cells. Blood group O buffy coats released significantly higher amounts of IL-6, TNF and nitric oxide compared with leukocytes from group A donors.

7.6.2 Susceptibility of blood group A to gastric cancer

Chronic infection and inflammation have been recognised as risk factors for a variety of human cancers and it has been proposed that active oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical generated in inflammed tissues can cause injury to target cells and also damage DNA. There is now increasing evidence to suggest that nitric oxide (NO) and its derivatives produced by activated phagocytes may also play a role in the multistage carcinogenesis process [reviewed by Ohshima and Barsch, 1994]. Ascorbic acid is thought to confer protection against neoplasia by scavenging nitrites, preventing the formation of potentially carcinogenic N-nitroso compounds and reactive oxygen metabolites, and by inhibition of the synthesis of DNA, RNA, and protein in tumour cells [reviewed by Goldstone *et al.*, 1996]. Gastric juice levels of ascorbic acid showed a significant rise after eradication of *H. pylori* infection [Sobala *et al.*, 1993]. When ascorbic acid scavenges reactive oxygen metabolites, ascorbyl radicals are generated with the increased release of reactive oxygen species in gastritis, higher concentrations of ascorbyl radicals are found in the gastric mucosa of subjects with *H. pylori* infection compared with control subjects [Drake *et al.*, 1995]

Direct evidence comes from the finding of enhanced gastric mucosal nitric oxide synthase activity in *H. pylori* positive patients [Rachmilewitz *et al.*, 1994] and nitric

oxide synthase gene expression in human macrophage lines is enhanced by soluble proteins from *H. pylori* [Chi *et al.*, 1994]. There were no published papers on the production of nitric oxide from human buffy coats in response to *H. pylori* ; therefore, part of this study was designed to examine the effect of whole cells of *H. pylori* , OMP and purified adhesin on the ability of human buffy coats to release nitric oxide. The results showed that both whole cells, OMP or purified adhesin induced nitric oxide and stimulation of human buffy coats with whole bacteria produced higher amounts of nitric oxide compared with purified adhesin or OMP. If nitric oxide plays a role in gastric cancer, these results suggested that the whole bacterium is the important factor for releasing higher levels of nitric oxide. The finding that lower concentrations of bacteria resulted in higher levels of nitric oxide production indicates that the association between *H. pylori* infection and gastric cancer might be related to lower density of colonisation. If the results of binding observed in an *in vitro* studies reflect colonisation *in vivo,* this might contribute to the increased susceptibility of blood group A individuals to gastric cancer.

7.6.3 Susceptibility of blood group A to ischaemic heart disease

Mendall and colleagues found that seropositivity for *H. pylori* conferred a twofold increased risk of coronary heart disease [Mendall *et al.*, 1994], and Morgando and colleagues observed an increase in the risk of myocardial infarction at younger ages for patients with *H. pylori* infection as judged by presence of antibodies in the blood [Morgando *et al.*, 1995]. This study supports previous observations that IHD patients had a significantly higher prevalence of *H. pylori* antibodies. It differs in that it examined levels of antibodies, and controlled for undiagnosed heart disease in the age and sex matched comparison group assessed in the analysis of results. These results are additional evidence that chronic infection with *H. pylori* might be a factor contributing to heart disease.

Epidemiological studies of IHD identified an association between blood group A and myocardial infarction and also with thrombosis [summarised by Mourant *et al.*, 1978]. The opposite was observed in this study; there were more blood group O individuals who died from of IHD compared with group A but this was not significant. The hypothesis that group A is more likely to survive from a heart attack and group O more likely to die needs to be examined with larger numbers of subjects.

H. pylori infection could contribute to increased risk of IHD by increasing fibrinogen levels [Patel *et al.*, 1994], and these bacteria also produce a tissue factor with procoagulant activity which can convert fibrinogen to fibrin [Miragliotta *et al.*, 1989]. The association between blood group A and susceptibility to IHD or disease involving clotting might be explained by the observation that people of blood group A have higher plasma levels of factor VIII (anti-haemophilic globulin) than do individuals of blood group O [reviewed by Mourant *et al.*, 1978] and the lower levels of nitric oxide observed with blood group A buffy coat responses to *H. pylori* might results in lower levels of vasodilator and reduction in desolution of clot formation or platelet aggregation.

If vitamins with anti-oxidant effects are involved in controlling damage due to the response of inflammatory cells to the bacteria, lower levels of vitamin C found in gastric juice of patients infected with *H. pylori* might contribute to the pathogenic process. These levels return to normal following eradication of the bacteria [Banerjee *et al.*, 1994]. The lower levels of vitamin C observed in the winter [Riemersma *et al.*, 1991] could contribute to decreasing the levels of anti-oxidant activity in plasma.

7.7 Future studies

Analysis of *H. pylori* isolates from different diseases (peptic ulcers, gastric cancer and IHD) for their abilities to stimulate buffy coats from different blood groups to release inflammatory mediators could further clarify host-parasite interactions underlying epidemiological observations.

There are a number of interactions between genetic and environmental risk factors associated with diseases in which *H. pylori* is implicated that need to be explained. Reactive oxygen radicals are thought to contribute to modification of serum lipids that could contributes to atherosclerosis [Carpenter *et al.*, 1995]. The lower levels of ascorbic acid observed in the gastric juice of patients infected with *H. pylori* [Banerjee *et al.*, 1994] needs to be considered in relation to infection and diet in the aetiology of both cancer and heart disease. Further studies are planned to assess levels of ascorbic acid in serum in relation to IHD and IgG levels to *H. pylori.*

Additional studies are needed to define the role of *H. pylori* in IHD or diseases involving clotting abnormalities and to determine if there is a correlation between *H. pylori* infection and coagulation factor levels.

The interaction between respiratory virus infections, *H. pylori* infection, dietary habits and fibrinogen levels need to be assessed in relation to the winter peak in deaths due to IHD.

Smoking is reported to reduce levels of prostaglandins which are major protective factors in gastric and duodenal mucosa against injury. *H. pylori* infected patients had low amounts of prostaglandin compared with non-infected patients [Goren *et al.*, 1989; Silecchia *et al.*, 1994]; therefore, further studies are required to determine if reduced prostaglandin levels are associated with increased binding and colonisation by *H. pylori* or with control of inflammatory responses.

If the adhesin that binds H type 2 and Lewis antigens is found on strains from different geographical areas, it could be considered as a candidate vaccine component to prevent *H. pylori* colonisation. Identification of the role of H type 2 and Lewis antigens as receptors for *H. pylori* also raises the possibility of anti-adhesin therapy with synthetic forms of these antigens for reduction and/or reversal of bacterial binding. If significant antibiotic resistance develops among *H. pylori* isolates this might become a major therapeutic approach.

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Study number:.....

NAME:.....

Hospital Number:.....

Date of birth:.....

Sex:.....

Address:.....

.....

Post code:.....

In the last month, have you had symptoms of:

1 Heartburn Yes/ No mild/ moderate / severe

2 Tummy pain Yes / No mild / moderate/ severe

3 Indigestion Yes / No mild / moderate / severe

What treatments has your doctor given to you in the past one year?

Antacids.....(Yes/ No)

Dosage...../ day

Cimetidine (Tagamet) .....(Yes / No)

Dosage.....mg/day

Ranitidine (Zantac).....(Yes / No)

Dosage.....mg/day

Omeprazole.....(Yes / No)

Dosage.....mg/day

What treatments are you taking now to relieve your symptoms?

..... Dosage...../day

Have you been on any antibiotic treatment over the past three months?

Yes / No

If yes to above, name the antibiotic .....

duration of treatment .....

condition treated for.....

Have you taken any painkiller in the past three months?

Yes / No

If yes, name the painkiller.....

dosage.....

condition treated for.....

Are you taking any painkiller regularly ?

Yes / No

If yes, name the painkiller.....

dosage.....

condition treated for.....

What other medical conditions are you known to suffer from?

.....

.....

.....

.....

Have you had an operation on you stomach ?

Yes / No

If yes, do you know what was done ?

.....

Are you taking low dose aspirin for heart condition or stroke?

Yes/ No If yes, dosage.....mg/day

Do you visit your dentist regularly?

Yes / No

If yes, please circle the following:

Every 6 months/ every year / every 2 year

When did you last have dental treatment?

.....

Do you have any dentures?

Yes / No If yes, is it full / partial ?

How often do you clean your teeth?

More than twice per day (Yes/ No)

Twice per day (Yes/ No)

Once per day (Yes / No)

Less than once per day (Yes / No)

Do you use dental floss regularly?

Yes / No

What is your job?

.....

Does your partner work ? (Yes / No)

If yes, what is his or her job?.....

Do you smoke? (Yes / No)

If yes,

How many cigarretes a day?.....

How many ounces of tobacco a day?.....

How long have you smoked for?.....

Are you an ex-smoker? Yes / No

If yes, when did you stop ?.....

How much alcohol do you drink in a week?

.....units

(a pint of lager is equivalent to 2 units; a glass of wine and a single liquer is equivalent to 1 unit each.)

INVESTIGATIONS

FBC: Hb.....

WCC.....

Platelets.....

MCV.....

Blood groups: ABO Type .....

Lewis Type .....

GGT:.....

ENDOSCOPIC FINDINGS:

Oesophagus:.....

.....

Gastric:(Ulcer is defined as lesion of at least 5mm in diameter with depth; specify size, number and location of ulcer if present.)

.....

.....

.....

Duodenum:.....

.....

.....

CLO: (Biopsy must be taken within 5 cm of antrum)

Positive/ Negative.

HISTOLOGIC FINDINGS:

(Two biopsy are each taken from gastric antrum, body and duodenum even if ulcer is absent. Biopsy must be taken from gastric ulcer if present.)

Body:.....

.....

Antrum:.....

.....

Duodenum.....

.....