Helicobacter pylori Infection

Pathophysiology, Epidemiology and Management

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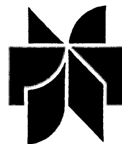

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Preface

Now that *Helicobacter pylori* is generally accepted as a key aetiological agent in gastric cancer as well as the main agent in peptic ulcer, it can claim to be the most important new discovery in clinical gastroenterology of the last decade, and yet there is no up-to-date book available on the subject that is designed primarily for the clinical gastroenterologist. This book aims to fill this gap. It should also be of interest to the basic scientist, to those providing a clinical laboratory service (microbiologists and histopathologists), and to epidemiologists and others involved in clinical research. There were over 900 publications on the subject of *Helicobacter pylori* last year, so no individual can hope to keep fully abreast of all aspects of the literature. The present book has been published within five months of the deadline for submission of manuscripts, in order to ensure that it is fully up-to-date at the time of publication.

The book contains 17 chapters by leading world authorities. It starts with a historical introduction by Professor Stewart Goodwin, within whose laboratory in Australia the organism was first cultured. It concludes with a conceptual overview by Professor David Graham, whose laboratory in Dallas, Texas, is currently the largest in the world involved in *H. pylori* research. It includes a chapter by Professor Martin Blaser from the USA which looks at pathogenesis at a molecular level; and one by Professor Guido Tytgat from The Netherlands who takes an overview of the role of *H. pylori* in peptic ulcer disease. Its role in gastric cancer is examined by Dr. Ashley Price (London) from the histopathological viewpoint, and by Dr. David Forman (Oxford) from the epidemiological viewpoint, including the large collaborative European study which he has coordinated. All aspects of diagnosis are covered. The current drug regimens are discussed by Dr. Tony Axon from Leeds, and the future role for vaccination in prevention and treatment by Professor Adrian Lee from Australia.

Tim Northfield

INTRODUCTION

1
Historical and microbiological perspectives

STEWART GOODWIN

INTRODUCTION

On 14 April 1982, in my Microbiology Department at Royal Perth Hospital, Western Australia, spiral bacteria from the human stomach were first successfully cultured. These are now called *Helicobacter pylori.*

In October 1989, at the second meeting of the European *Campylobacter* [*Helicobacter*] *pylori* study group in Ulm, Germany, I announced my publication that month of the new genus name *Helicobacter*, with the new species names *Helicobacter pylori* and *Helicobacter mustelae*[1]. However, the origin of the name *Helicobacter* started 2 years earlier, in 1987, when I had been in correspondence with Professor MacAdoo, of the Virginia Polytechnic Institute. He suggested three possible names for the new genus: *Gastronosobactrum*, *Ventrimorbibacter*, and *Helicobaculum.* I combined two of these suggestions and designed the name *Helicobacter* in December 1987.

EARLIEST PAPERS

Credit for the first description of spiral organisms in the human stomach should probably be given to Bottcher in 1874[2]. Bizzozero in 1893[3], and Salomon in 1896[4] described 'spirochaetes' in animals, but these were unlikely to have been *H. pylori.* Probably they were '*Gastrospirillum hominis*', which has not been cultured or officially named. Krienitz described human gastric spiral organisms in 1906[5], Doenges in 1938[6] and Freedberg and Barron in 1940[7]. Those who described spiral bacteria in humans and animals usually failed to distinguish the shorter, gently spiralled *H. pylori* from the longer, tightly spiralled '*G. hominis*'[6].

Studies of human *H. pylori* were at first only histological and ultrastructural, without successful culture. In 1975 Steer noted that spiral bacteria were closely apposed to the gastric mucus-secreting cells[8], but culture yielded only *Pseudomonas aeruginosa*[9]. Electron micrographs of spiral bacteria in large

numbers on gastric epithelial cells were published by him in May 1984[10]. Independently, in Birmingham, England, from 1981 Rollason *et al.* had observed gastric spiral bacteria[11].

FIRST CULTURE OF *H. PYLORI* IN 1982

In Perth, Western Australia, at the Royal Perth Hospital, histological and ultrastructural studies of the gastric mucosa had been published in 1979[12]; spiral bacteria were seen, but because they did not invade the mucosa were thought to be irrelevant. The histopathologist, Warren, correlated them with the presence of polymorphonuclear leucocytes[13]. In 1981, Marshall was training in internal medicine, and for 6 months was learning gastroenterology. With Warren he reviewed the patients in whom large numbers of gastric spiral bacteria had been seen[14]. One of these had been treated fortuitously with tetracycline; his symptoms resolved, and subsequent endoscopic biopsy showed that the antral gastritis had also resolved[14].

It was most fortunate that by then Armstrong had come to Royal Perth Hospital from Mill Hill to head the Electronmicroscopy Unit. He obtained high-magnification electron micrographs of *H. pylori* in biopsy material. I was Head of the Microbiology Department, and in late 1981 Marshall asked me for microbiological assistance. A protocol was agreed (which I still possess); gastric biopsy specimens were to be obtained from 100 consecutive patients. They would be taken by the consultant gastroenterologists, Waters and Sanderson, and would be processed both in the Microbiology Department by Gram stain and culture, and in the Histopathology Department. The project started in March 1982, and I asked my microbiologist colleague, Pearman, to supervise the project. Among the first 34 specimens, spiral bacteria were seen in the Gram stain in six. However, in spite of frequent variations of media, and incubation temperatures, spiral bacteria were not cultured, because incubation was limited to 48 h. The thirty-fifth culture was left incubating during the Easter holiday, which in Australia lasted for 5 days. When the plates were finally viewed, a pure growth of 1 mm transparent colonies was seen. *H. pylori* had finally been cultured! The date was 14 April 1982[15]. Gram stain of the colonies showed only slightly curved organisms, not spirals as in the smear of the specimen, and Marshall doubted whether we had grown the correct organism. From subsequent specimens 11 isolations of this new organism were achieved. Among the 100 specimens, spiral bacteria were seen in the Gram stain in 34. Histologically, spiral bacteria had been seen in 58 patients. The culture of *H. pylori* was not an accident, as stated by some; but it was fortunate that in Perth a 5-day holiday occurred during the project, and so demonstrated that 'chance favours the prepared mind'.

At this time we all became indebted to the skills of Annear, the senior scientist in our Microbiology Department, who successfully maintained these cultures. From his broth cultures Armstrong and Wee produced electronmicrographs that revealed that the bacteria were spiral, and had five sheathed flagella, which indicated that they were not *Campylobacter jejuni*. Annear also achieved lyophylization of several cultures of *H. pylori* in May

1982. The two earliest isolates are now NCTC 11637 and NCTC 11638.

Marshall quickly realized that gastritis was highly associated with duodenal ulcer (DU) and gastric ulcer (GU). In the Perth study, 100% of patients with DU and 80% of those with GU had these spiral bacteria[14]. In 1983 Marshall moved to Fremantle Hospital, and wrote the first published description of the culture of *H. pylori*[16]. When Warren and Marshall could not agree on the wording of a joint letter to the *Lancet* in 1983, Armstrong advised them to write separate letters[13,16]. An early reference to the culture of *H. pylori* was an 1983 editorial on the International Workshop on *Campylobacter* infections, held in Brussels[17], when the name *Campylobacter pyloridis* was suggested by Skirrow. The name *C. pyloridis* was formally proposed in 1984[15]. However the rules of Latin grammar required us to change the species name to *C. pylori*[18].

In June 1984 Marshall and Warren's article on the first culture of 'unidentified curved bacilli' appeared[19], and also Langenberg's letter from Amsterdam detailing the powerful urease enzyme of this organism[20]. During 1983 Marshall devised selective media for primary isolation, and discovered the sensitivity of *H. pylori* to bismuth and metronidazole; in 1985 he reported this in a detailed study of 267 patients[21]. In 1986 I reviewed our knowledge up to that time, with Armstrong and Marshall, and started with the words 'Until a microbe is cultured and characterised, histopathological observation of the new organism remains tantalisingly incomplete'[22].

REALIZATION THAT THESE HUMAN SPIRAL BACTERIA WERE A NEW GENUS

During 1983 I was on sabbatical leave from Perth, but in January 1984 I inserted *H. pylori* into our study of cellular fatty acids, and was thrilled to discover a unique profile which indicated a new genus. Armstrong and I suggested this when we published these findings with his ultrastructural studies[23]. We continued our study of the basic biology of *H. pylori*, and discovered that thermoplasmaquinones were absent in *H. pylori*, which is an important taxonomic feature[24]. By DNA–DNA hybridization Terry Chilvers detected that *H. mustelae* should not be a subspecies of *H. pylori*, but was a unique species[25]. Others had studied the sequence of RNA in ribosomes, and concluded that *H. pylori* was not in the *Campylobacter* genus (see below). By 1988 our Perth microbiology team and international colleagues had accumulated sufficient evidence to justify a new genus, for which I devised the name *Helicobacter*[1]. At the Fifth International *Campylobacter* Workshop in Mexico in February 1989 the *Campylobacter* taxonomy committee agreed that *H. pylori* should not be in the *Campylobacter* genus. In June the *International Journal of Systematic Bacteriology* accepted our paper creating the new *Helicobacter* genus, and it was published in October 1989. So we now have *H. pylori*, which is unlikely to change yet again!

OTHER DISCOVERIES ABOUT *H. PYLORI*

From the historical perspective it is worth noting when other discoveries were first made, although authors from North America and even Eastern Australia consistently fail to refer to them. Many occurred before these later workers became interested in *H. pylori.* Thus our Perth team made the first evaluation of different methods of processing biopsy material for *H. pylori*[26], and at the same time we were the first to note that the inflammation of *H. pylori* is patchy. We were the first to describe an ELISA assay for *H. pylori* antibody[27], and the first to publish a study of *H. pylori* in children[28]. The discovery of genomic variation, as shown by restriction endonuclease analysis of *H. pylori* DNA, I made with Suzanne Majewski[29], but a recent review of genomic variation of *H. pylori* (from Sydney) in the *Lancet* failed to record this[30]. The first descriptions of plasmids in *H. pylori* contained the spelling error *C. pyloridi* which slipped through to the journal while I was on sabbatical leave[31]. With Armstrong and Wee we reported the ultrastructural appearances of the effect of antibiotics on *H. pylori*[32]. John Armstrong was the first to note adherence pedestals in the gastric mucosa with *H. pylori* inflammation[33]. Mark Taylor of St Mary's London and I were the first to suggest that the urease of *H. pylori* had two pH optima[34]. Barry Marshall devised the rapid urease test in the endoscopy room, and we compared this with detection of *H. pylori* by microbiological methods[35]. With Cheryl McCullough and John Boehm I described the optimal methods for preservation of helicobacters and campylobacters[36], and with Jennifer Robinson the discovery of soluble haemagglutinins of *H. pylori*[37].

RNA SEQUENCING AND OTHER TAXONOMIC FEATURES

Primary taxonomic criteria to distinguish genera and species must be genomic, DNA and RNA. 5S and 16S ribosomal ribonucleic acid sequencing first showed that *H. pylori* did not belong in the *Campylobacter* genus[38]. These early studies also indicated a certain affinity between *H. pylori* and *Wolinella succinogenes.* However, an extensive study of 14 phenotypic differences outweighed the genomic relatedness of these organisms[39], and subsequent work by Vandamme *et al.* has again separated *Helicobacter* from *Wolinella*[40]. Our first description of the *Helicobacter* genus cited five major, distinguishing groups of taxonomic features – ultrastructure, cellular fatty acid profiles, respiratory quinones, growth characteristics, and enzyme capabilities[1].

The main morphological feature of the *Helicobacter* genus is the possession of sheathed flagella, and in nearly all members there is also a membranous terminal bulb, which is an extension of the sheath[1]. *H. pylori* has four to six unipolar flagella, each 2.5 μm long and about 30 nm in thickness. The cell wall membrane is smooth is comparison with the rugose membrane of *C. jejuni*[1]. On the human gastric mucosa, *H. pylori* is curved or gently spiral, but when cultured *in vitro* true spiral forms may be few or absent. Jones and Curry first reported that prolonged culture gives rise to the emergence of coccoidal forms[41].

Our studies in Perth of the external surface of *H. pylori* using tannic acid revealed that the organism has a distinctive glycocalyx up to 40 nm in thickness, which *in vivo* often coalesces with the glycocalyx of the mucosal cells where there are adherence pedestals[1]. *H. pylori* are also covered with ring-like subunits 12–15 nm in diameter[1].

UREASE

A useful summary of the history of gastric urease has been given by Hazell and Mendz[42], where specific references can be found. The presence of urease in the stomachs of carnivorous animals and ruminants was first demonstrated by Luck in 1924. In dogs, 'gastric urease' was essentially the same as soybean urease. Luck and Seth proposed possible functions for 'gastric urease'; including secretion of alkali to control gastric hyperacidity, an intercellular mechanism for mucosal protection against gastric acidity, and regulation of stomach wall movement through the action of ammonium bicarbonate on muscle fibres, or as a specific property of ammonia ions. Hollán in 1947 found urease in the stomachs of humans, as did Glick (1949), who considered that the enzyme appeared to vary directly with the ability of the stomach to secrete acid. Von Korff *et al.* found that in dogs the gastric ammonia concentration was a function of plasma urea, and this did not affect significantly gastric juice acidity. Fitzgerald and Murphy proposed that peptic ulceration was 'probably due to the ill-adjusted interplay of secretion, neutralization and a third factor – mucosal resistance'. They found that in many subjects given urea, either orally or by injection, the rise in gastric ammonia was quite small, yet a marked fall in the level of gastric acidity was recorded. Kornberg's group established that 'gastric urease' was of bacterial origin, a result that was later confirmed by Laverson *et al.* in germ-free rats. However, in the cat, urea hydrolysis did not occur in non-secreting stomachs despite the presence of urea-splitting bacteria. It is easy to see how early investigators in this area could have considered urease to be a regulatory enzyme required to maintain mucosal integrity.

In the *Helicobacter* genus most of the named species possess potent urease activity which provides a primary biochemical distinction from other oxidase- and catalase-positive spiral organisms. However, *H. cinaedi* and *H. fennellie* are urease-negative. The urease enzymes of gastric helicobacters (Table 1) possess two pH optima, including one at acid pH[34,43]. The latter is absent from the urease of lower gut pathogens, such as *H. muridarum*[43]; urease activity at low pH may be essential for the colonization of gastric mucosa. Molecular studies of urease by Labigne are reviewed below.

CELLULAR FATTY ACID COMPOSITION

In 1985 *H. pylori* was reported by us to exhibit a new profile of cellular fatty acids characterized by relatively large amounts of tetradecanoic (14:0),

Table 1 *Helicobacter* species and their hosts (as of 1993)

Species	*Hosts*	*Primary site*
H. pylori	Human (?monkey, ?pig)	Stomach
H. mustelae	Ferret	Stomach
H. felis	Cat, dog	Stomach
H. nemestrinae	Pig-tailed macaque monkey	Stomach
H. acinonyx	Cheetah	Stomach
H. muridarum	Mice and rats	Intestine
H. cinaedi	Humans and rodents	Intestine
H. fennelliae	Humans	Intestine
'*H. rappini*'	Sheep, dogs, humans	Liver, stomach, intestine

octadecanoic (18:0) and 19-carbon cyclopropane (19:0 cyc) fatty acids[23]. *Campylobacter* spp. by contrast are relatively devoid of 18:0 and contain very small amounts of 14:0. *H. pylori* was then found to be unique in possessing 3-hydroxyocta-decanoic (3-OH-18:0), leading Lambert *et al.*[44], to define the pattern as a new GLC group G.

METABOLISM OF *H. PYLORI*

Up to now it has been stated that *H. pylori* does not catabolize saccharides. However, Hazell and Mendz have discovered highly specific monosaccharide kinases in *H. pylori* by monitoring phosphorylated products using ^{13}C or ^{31}P NMR[42]. They found evidence for the presence of enzymes of the pentose phosphate pathway in *H. pylori*, and they detailed the pathway for the utilization of glucose by *H. pylori*[42].

MOLECULAR STUDIES ON *H. PYLORI*

The most significant early work on *H. pylori* was done by Labigne, joined recently by Ferrero, in Paris. Labigne created a shuttle cloning system which allowed expression of *H. pylori* urease in *C. jejuni*. Her shuttle cloning vector was capable of allowing the mobilization of DNA fragments between *E. coli* and *C. jejuni* having been modified by the introduction of a 'cos' site. Large fragments of *H. pylori* chromosomal DNA, partially digested with restriction enzyme, were cloned into this new cosmid vector. Transduction of *E. coli* bacteria with bacteriophage lambda particles carrying the recombinant cosmids resulted in the creation of a genomic library of *H. pylori* DNA. Mobilization of 106 recombinant cosmids from *E. coli* to *C. jejuni* led to the isolation of a *C. jejuni* transconjugant which was capable of weakly hydrolysing urea[45]. Labigne and Ferrero have published linear restriction maps of the recombinant cosmid and plasmids they have used, showing the location of the important urease genes from *ureA* to *ureH*. They have specified the genes essential for *H. pylori* urease expression in *E. coli* and identified non-essential genes[45]. They have discussed each of these genes in detail and the construction of urease-negative *H. pylori* mutants, and the

polymorphism of the urease region and its use for the molecular typing of *H. pylori* isolates[45].

The genome of *H. pylori* is of fundamental significance for an understanding of the organism, and a genome map has been constructed by Taylor using pulsed-field gel electrophoresis of *H. pylori* chromosomal DNA[46].

PROTEINS OF *H. PYLORI*

A vacuolating cytotoxin of *H. pylori* was originally detected by Leunk *et al.*[47], and this may be of significance because it seems to occur more frequently in strains associated with duodenal ulcer than in strains of *H. pylori* isolated from patients only with gastritis. A useful summary of the many proteins so far identified in *H. pylori* has made by Dunn[48]. He used two DGE silver-stain preparations of soluble, whole cell proteins to separate various proteins, and in this way has identified some of them. He has shown that a component of *H. pylori* flagellin crossreacts with the flagellin of *C. jejuni.*

RECEPTORS AND ADHESINS

Adherence of *H. pylori* to the gastric mucosa is a fundamental stage of infection. If it is understood in detail then it is an obvious site of attack for a vaccine. If adherence can be prevented then infection can be prevented. Lingwood *et al.* have done seminal work on the identification of *H. pylori* receptors[49]. They have screened human erythrocytes and human gastric mucosa for the presence of *H. pylori* binding receptors. By HPLC and GLC analysis they have determined that the *Helicobacter* glycerolipid receptor is a species of phosphatidyl ethanolamine (PE). In addition to PE they have detected a second *H. pylori* binding specificity, for gangliotetraosyl and gangliotriaosyl ceramide (Gal*beta*1-4GalNac*beta*1-4Gal*beta*1-4 glc ceramide-Gg_4 and GalNac*beta*1-4Gal*beta*1-4 glc ceramide-Gg_3, respectively). Although these glycolipids are not found in significant concentrations in the human antrum, it is possible that high levels are expressed in a minor subset of gastric epithelial cells. Lingwood *et al.* have also discovered that exoenzyme S from *Pseudomonas aeruginosa* binds in an identical fashion to the *Helicobacter* glycerolipid receptor isolated from the human stomach. This is an ADP ribosyl transferase.

DUODENAL ULCER

It was initially difficult to understand how *H. pylori* might cause damage within the duodenum, but this is explained by the presence of patches of gastric metaplasia in the duodenum of patients with duodenal ulcer, which was first reported by Wyatt *et al.*[50], who indicated that this gastric type of tissue would be colonized by *H. pylori* with resulting duodenitis. In an effort to highlight the difference between the predisposing cause of duodenal ulcer

which is *H. pylori* duodenitis and the precipitating cause which is acid and pepsin, the 'leaking-roof' concept was enunciated[51]; together with the new dictum 'Cure the duodenitis and the duodenal ulcer will look after itself'. This led on to antibiotic treatment of duodenal ulcer which will be reported separately in this volume.

ANIMAL MODELS FOR *H. PYLORI* GASTRITIS

Five different groups of animals have been used to simulate *H. pylori* infection either with human isolates of *H. pylori* or with other gastric *Helicobacter*. These have been reviewed by Fox and Lee[52]. The ferret is infected with *H. mustelae* to induce gastritis very similar to *H. pylori* gastritis. The gnotobiotic piglet is a useful model for the induction of gastritis and has been used to identify virulence factors required for initiation of infection. The mouse can be infected with '*G. hominis*' and *H. felis* to produce significant infections which may be very useful for the assessment of new antibiotics to eradicate gastric *Helicobacters*. These rodent models can also be useful to test vaccine developments, although much more sophisticated molecular biology will probably need to be done first[53].

CONCLUSION

Many important discoveries in the history of *H. pylori* have not been mentioned in this brief review, because they will be referred to in other chapters.

SUMMARY

The discovery of *H. pylori* in Western Australia has revealed that this organism causes a chronic human infection which is the most widespread such infection in the world. Naturally, many of the early discoveries about *H. pylori* were made in Western Australia, and these have been delineated, together with other biological and clinical landmark discoveries about *H. pylori*.

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2
Geographic distribution and association with gastric cancer

DAVID FORMAN and PENNY WEBB

INTRODUCTION

This chapter reviews the distribution of *H. pylori* infection throughout the world, and its association with socioeconomic development. A comparison is then made with the pattern for gastric cancer, the only *H. pylori*-associated disease for which there is reliable information about geographic distribution.

GEOGRAPHIC DISTRIBUTION OF *H. PYLORI*

The geographic distribution of *H. pylori* infection is primarily associated with economic development. In general, infection rates decrease with improvements in socioeconomic conditions, a relationship that presumably reflects changes in lifestyle which influence acquisition of the bacteria. The prevalence of infection is thus considerably lower in developed countries than in developing countries, an effect that is particularly pronounced in childhood and early adult life. Figure 1 shows the contrast between the developing and the developed world in the prevalence of *H. pylori* infection at different ages. In developing countries the prevalence of infection rises steeply soon after birth and may reach levels of 80–90% by the age of 20 years. The prevalence remains at this level for the rest of adult life. In developed countries infection is relatively uncommon (less than 20%) below the age of 25–30 years. The prevalence then increases gradually with age at a rate estimated to be approximately 1% per annum[1]. Above about 70 years the prevalence appears to increase more slowly, and to level out at around 60–70%. Although there are important exceptions to this general pattern (discussed below), it is evident that the burden of *H. pylori* infection is far greater in the developing than in the developed world.

In support of the age–prevalence profiles presented in Fig. 1, Figs 2 and 3 show data from some of the larger prevalence studies in five countries (Peru[2], Thailand[3], Japan[4], the UK[5], and the US[1]). In each of these studies

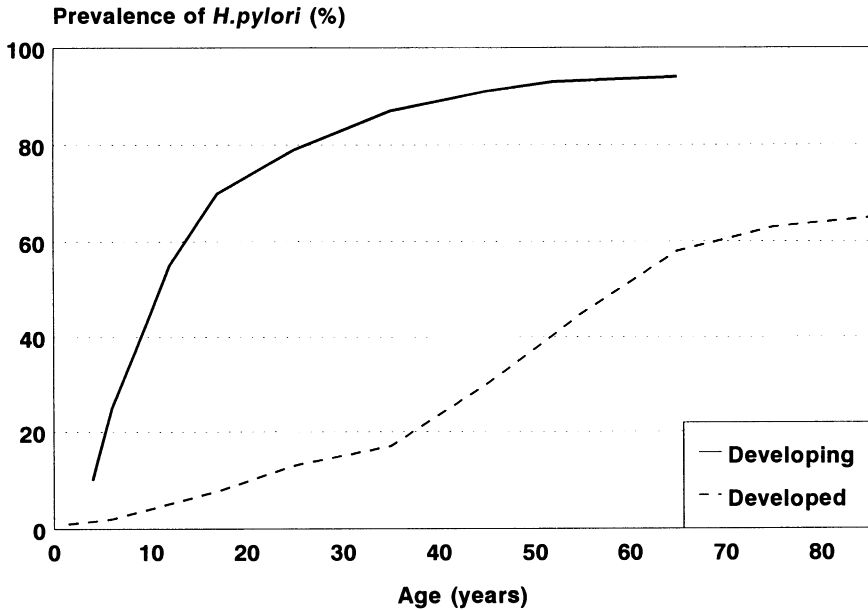

Fig. 1 Prevalence of *H. pylori* infection by age: developing vs developed world

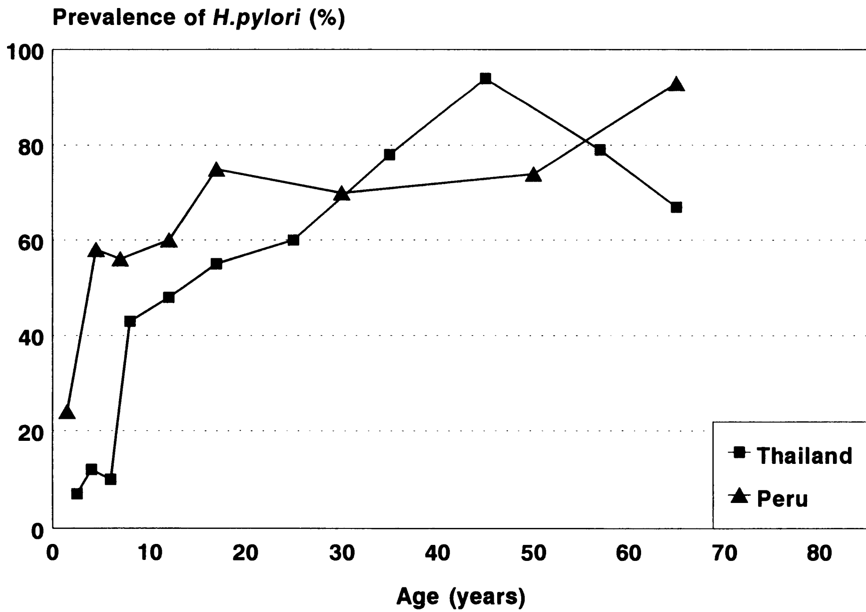

Fig. 2 Prevalence of *H. pylori* antibodies by age: developing countries. Redrawn from the Gastroenterology Physiology Working Group[2] and Perez-Perez *et al.*[3]

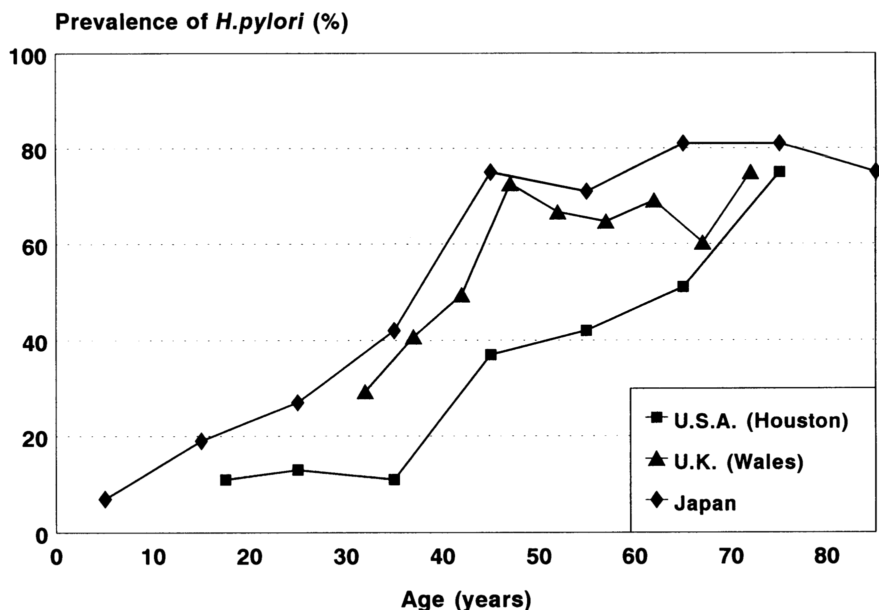

Fig. 3 Prevalence of *H. pylori* antibodies by age: developed countries. Redrawn from Graham *et al.*[1], Asaka *et al.*[4] and Sitas *et al.*[5]

the presence of infection was determined using an ELISA to detect IgG antibodies to *H. pylori* in blood samples[6]. The results are all highly consistent with the dichotomy of developing vs developed countries presented in Fig. 1, although there is heterogeneity in the age–prevalence profiles within both the developing and the developed world. For example, Fig. 3 shows that, at all ages up until 60 years, the prevalence in the US is substantially lower than that in the UK or in Japan. A study carried out in 46 different counties within rural China[7] has shown considerable variation between counties, all with relatively low levels of development, with adult prevalence rates ranging from 28% to 96%. Thus, there will be exceptions to the general pattern presented in Fig. 1 and this will have a bearing on the associated geographic pattern of disease. Nevertheless, most published data are in accord with the scheme shown in Fig. 1[8,9].

It is important to bear in mind that seroprevalence data presented by age, as in Figs 1–3, do not provide complete information about the age of acquisition. Even though the seroprevalence pattern in developed countries indicates current low levels of infection in children and young adults, this does not mean that childhood acquisition is a rare event in these countries. On the contrary, childhood may be the dominant period for acquisition in both developing and developed countries, and those who have current adult infections may have acquired them as children many years previously. This would imply that, in the developed world, there has been a gradual reduction in childhood acquisition over time, giving rise to the observed association with age.

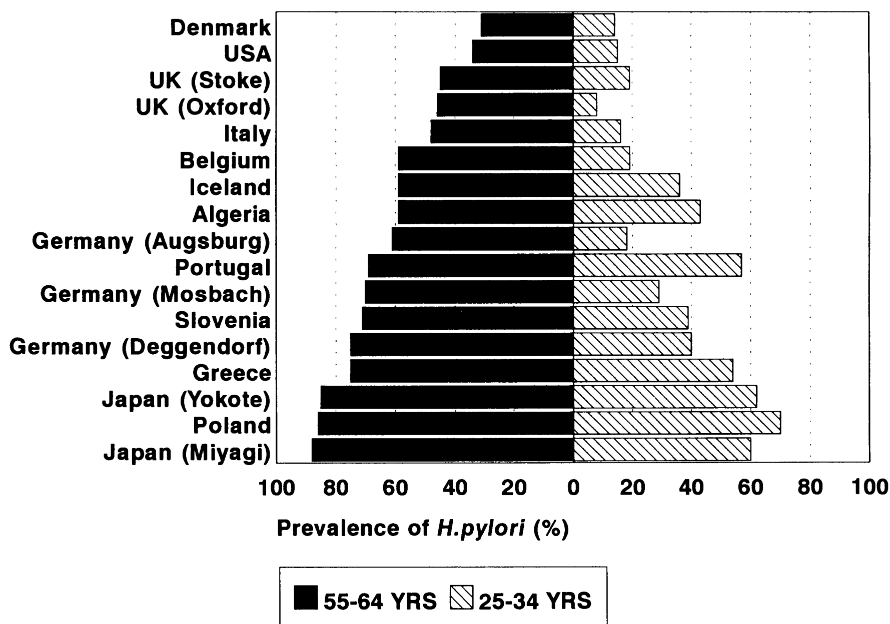

Fig. 4 Prevalence of *H. pylori* antibodies at 55–64 years and 25–34 years; 17 Eurogast centres

When the focus of interest shifts from the global perspective to more detailed comparisons between countries, a number of methodological issues become important. It is critical to compare similar cross-sections of the population in each country and not, for example, to compare blood donors in one population with clinic attenders in another. The blood used for serological testing should be processed and stored in an identical manner and a standardized assay should be used for all samples. These criteria have been met in the Eurogast study[10] in which *H. pylori* antibody prevalence was compared in 17 populations drawn from 13 different countries. In each population approximately 50 males and 50 females in each of two age-groups, 25–34 and 55–64 years, were selected at random from local population registers. Blood was collected using a standardized protocol and assayed, in a single laboratory, using a validated ELISA[6].

H. pylori antibody prevalence rates from Eurogast are shown in Fig. 4 for the two age groups: 25–34 years and 55–64 years. In the younger age group there was a nine-fold variation in prevalence (from 8% in the Oxford, UK, centre to 70% in Poland) while in the older age group there was a three-fold variation (from 31% in Denmark to 87% in both Poland and the Miyagi, Japan, centre). Prevalence rates tended to be lower in the populations from the US and northern Europe compared with higher rates in southern and eastern Europe and in Japan. The pattern is broadly consistent with higher prevalence rates being found in those countries with either relatively poor economies or with economies that developed more recently.

As expected, the prevalence in each Eurogast population was higher in the older age group than in the younger group, although there was a strong correlation between the prevalence at 25–34 years and that at 55–64 years ($r = 0.88, p < 0.001$). There were, however, some populations with a relatively low prevalence at 25–34 years but a relatively high prevalence at 55–64 years, the populations from Germany being notable in this respect. If the prevalence rates in different populations are determined by underlying socioeconomic conditions, then populations such as those in Germany which show a relative change in prevalence at different ages may have undergone major socioeconomic transformations that have affected only the younger generation.

An obvious issue for future research will be the identification of the specific social factors that influence bacterial transmission and, hence, the geographic distribution of *H. pylori*. If, as has been suggested[11,12], overcrowding especially in early childhood, is of particular relevance to transmission, then it will be important to consider the time period when economic changes occur and how they affect living density. Thus countries which have undergone economic transformations, but only in the past 25 years, or where domestic crowding has remained high, may still have high adult prevalence rates. Either, or both, of these possibilities may explain the high prevalence rate seen in the younger age group in countries such as Japan.

GASTRIC CANCER AND *H. PYLORI*

There is increasing evidence to suggest that infection with *H. pylori* is a risk factor for gastric cancer. Prospective studies from three populations in the developed world have shown that prior infection with *H. pylori* confers a 3–6-fold increased risk of gastric cancer[13–15]. Because of the high prevalence of *H. pylori* within adult populations, a high proportion of cancers could be attributable to the infection.

Gastric cancer is the only disease associated with *H. pylori* infection for which there is reliable information about geographic distribution. As with *H. pylori* infection, this cancer is much more common in developing countries than in developed countries[16]. In the developed countries, incidence and mortality rates are declining rapidly[16,17], and this decline has been occurring for longest in countries with well-developed economies such as the US and northern Europe. A geographic association between gastric cancer rates and the prevalence of *H. pylori* infection might therefore be anticipated. Several studies have attempted to look formally at this relationship. Four studies[18–21] have compared regions with high and low gastric cancer risk within a single country and looked at the corresponding antibody prevalence levels in these regions (Table 1). Two of these studies, in Colombia[18] and China[19], showed significant results with a higher prevalence in the high-risk region whereas the other two, in Costa Rica[20] and Italy[21], showed no difference in prevalence between the regions. In the Chinese study[19], the significant difference was observed only when comparing antibody prevalence rates in young children under 5 years old, consistent with the view[22,23] that high

Table 1 Geographic comparisons of *H. pylori* antibody prevalence rates and gastric cancer risk: within-country studies

	H. pylori *prevalence* (%)			
Country	*Reference*	*High risk*	*Low risk*	
Colombia	18	93	63	$p = 0.01$
Costa Rica	20	66	72	n.s.
Italy	21	44	45	n.s.
China (under 5 years)	19	51	23	$p < 0.05$

cancer risk is determined by acquisition of *H. pylori* at an early age.

There have been two more extensive geographic studies of the relationship between *H. pylori* infection and gastric cancer. The study, referred to above, of 46 counties in rural China[7] showed a significant correlation ($r = 0.40$, $p = 0.02$) between the antibody prevalence rate and gastric cancer mortality, while the Eurogast study[10] showed a significant regression of cancer mortality on antibody prevalence ($p = 0.002$). Although geographic associations are considered to be a weak form of epidemiological evidence, the fact that these two large studies have both produced statistically significant positive results adds weight to the hypothesis that *H. pylori* infection may partly explain the geographic pattern of gastric cancer.

Examination of the plots from the two studies (Figs 5 and 6) shows that, despite statistical significance, there was considerable scatter around the line of best fit. In China there were a number of counties with extremely high antibody prevalence rates but low cancer rates, a situation also seen in the Eurogast centre from Greece (Crete). In Eurogast there were also populations with low antibody prevalence rates and high cancer rates, notably in Italy (Florence) and the UK (Stoke). Finally, in both studies there were populations with widely differing antibody prevalence rates but similar cancer rates. Gastric cancer has a multifactorial aetiology and it is certain that risk factors apart from *H. pylori* infection, notably dietary behaviour, are involved in its causation. Thus one may expect to find populations with high rates of *H. pylori* infection that do not have a high risk of gastric cancer because of a favourable diet. A further problem in this type of geographic study is that current antibody prevalence rates in middle-aged adult populations are compared with cancer rates for periods of time some years in the past, that will be dominated by the rates in the elderly. Populations which have undergone major socioeconomic changes may therefore have a current prevalence of infection very different from the previous generation which contributed most to the cancer risk. Thus, while *H. pylori* infection may be related to gastric cancer risk when comparing populations with a wide range in cancer rates, it is perhaps unrealistic to expect the association to persist when comparing populations within a narrow range. This may be the situation in the Costa Rican[20] and Italian[21] studies in which no regional differences in prevalence were seen.

Further interesting observations, of relevance to the geographic association between *H. pylori* infection and gastric cancer, derive from the studies of Barker and colleagues[24,25]. They have examined the pattern of gastric cancer

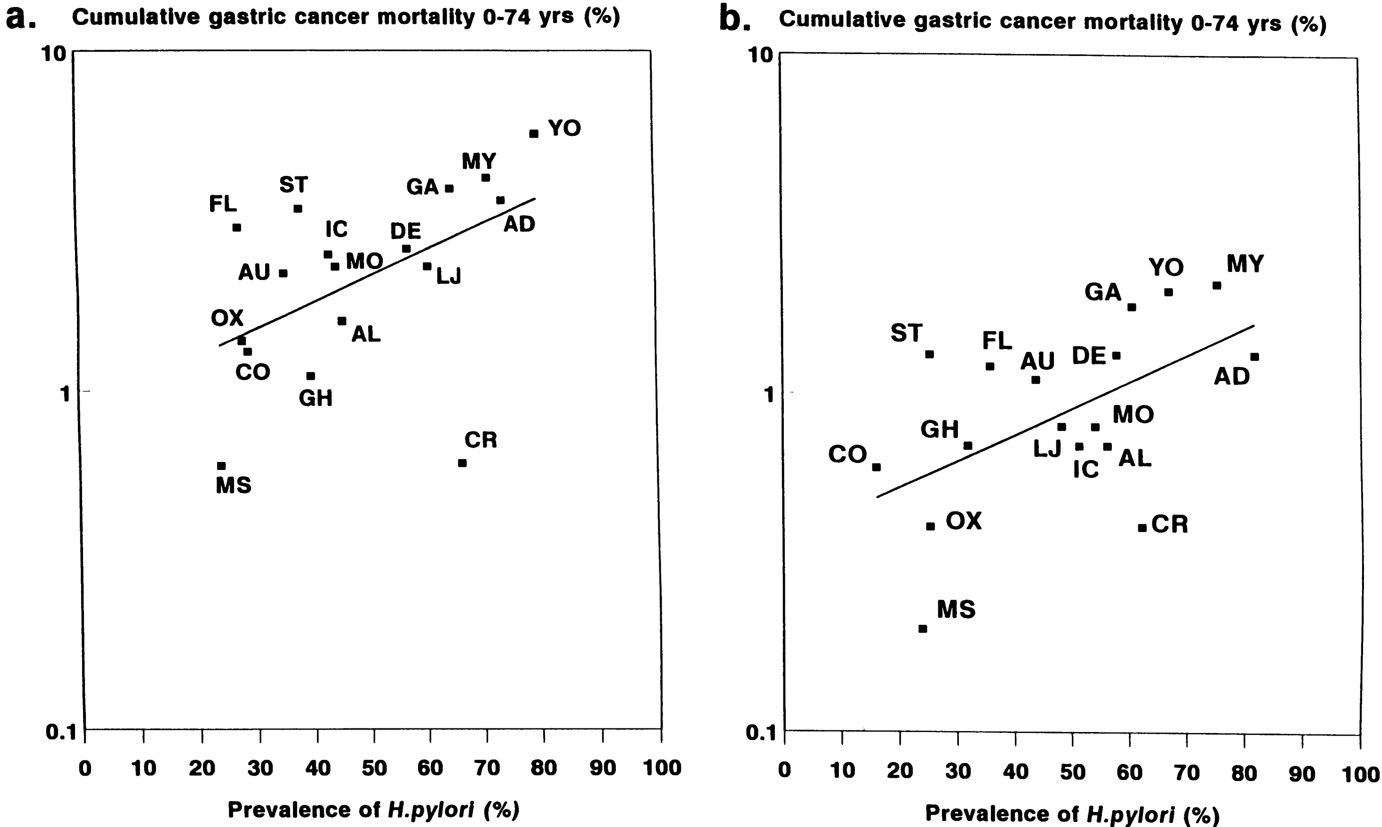

Fig. 5 Gastric cancer mortality by *H. pylori* antibody prevalence; Eurogast study: (**a**) = males, (**b**) = females. Redrawn from Eurogast study group[10]. Centre codes: AL = Algiers, Algeria; GH = Ghent, Belgium; CO = Copenhagen, Denmark; AU = Augsburg, Germany; DE = Deggendorf, Germany; MO = Mosbach, Germany; CR = Crete, Greece; IC = Iceland; FL = Florence, Italy; MY = Miyagi, Japan; YO = Yokote, Japan; AD = Adamowka, Poland; GA = Gaia, Portugal; LJ = Ljubljana, Slovenia; OX = Oxford, UK; ST = Stoke, UK; MS = Minneapolis–St Paul, USA

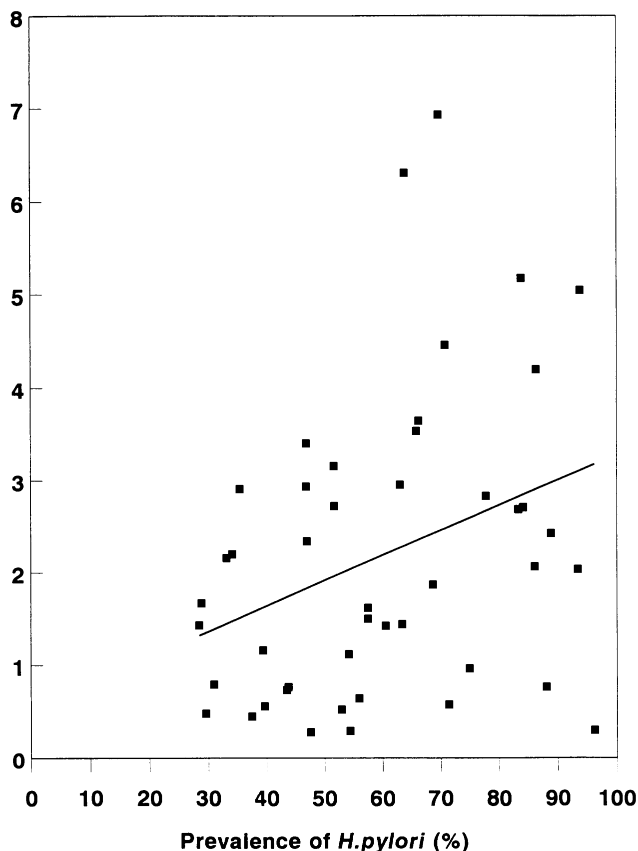

Fig. 6 Gastric cancer mortality by *H. pylori* antibody prevalence; 46 rural counties in China. Redrawn from Forman *et al.*[7]

mortality throughout the standard 212 local authority areas of England and Wales. They found first that the cancer mortality for the period 1968–78 in migrants, i.e. those who moved from one part of the country to another, was associated much more strongly with place of birth than with place of death. This confirms older studies in migrants showing that early life environment is an important determinant of the geographic variation of gastric cancer. Barker *et al.* then went on to show that gastric cancer mortality in 1968–78 was significantly correlated with the level of overcrowding in the area, as assessed by a detailed government survey carried out in 1936. No other specific cause of death in the 1968–78 period was as strongly associated with the 1936 level of overcrowding as gastric cancer and overcrowding in later time periods, assessed at censuses in 1951 and 1971, correlated less well with gastric cancer than the measure used in the 1936 survey. Overcrowding in 1936 was also strongly correlated with post-neonatal mortality in the period

1931–35, and particularly with specific post-neonatal causes of death known to be infectious in origin — bronchitis, measles and diarrhoea. These data are all consistent with a role for early life environment, especially childhood-acquired infections such as *H. pylori*, being partly responsible for the subsequent risk of gastric cancer.

SUMMARY

The geographic distribution of *H. pylori* is closely related to levels of socioeconomic development. The prevalence of infection is significantly higher in developing countries than in developed countries, an effect particularly pronounced in childhood and early adult life. There are important variations in the prevalence of infection within both the developing and the developed world. Gastric cancer shows a similar pattern of geographic distribution to *H. pylori* infection and two large studies have shown a statistically significant relationship between cancer risk and the prevalence of *H. pylori* antibodies. There are, however, some exceptions to this association that arise from the multifactorial aetiology of gastric cancer and from the time discrepancy involved in comparing current infection rates with past cancer rates. In England and Wales, geographic studies have shown that gastric cancer is strongly related to overcrowding, especially when this is assessed several decades earlier. This is consistent with the involvement of an early life infection, such as *H. pylori*.

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EPIDEMIOLOGY

3
Natural history and mode of transmission

MICHAEL MENDALL

INTRODUCTION

H. pylori infection is one of the world's most common bacterial infections. *H. pylori* clearly is not a commensal organism, as it is associated with chronic active inflammation in the stomach, peptic ulcer disease and probably gastric cancer.

The study of the mode of transmission is made difficult by the fact that, unlike other infectious diseases, there is no well-defined clinical syndrome associated with its acquisition. Hence, the usual approach with infectious diseases, of identifying cases and determining important exposures, is not possible.

Most epidemiology has been performed using serology for diagnosis with varying antigen preparations. The findings in the limited number of studies using the ^{13}C urea breath test and endoscopy have supported the conclusions of these studies.

PREVALENCE OF INFECTION

H. pylori infection is more prevalent in the developing world, seroprevalence rising rapidly after 1 year of age, with half the population infected by the age of 10. By young adulthood more than three-quarters of the population is seropositive. In the developed world the picture is different, there being a gradual rise in seroprevalence through life, reaching 40–70% by old age[1–4]. The frequency of infection in children in the developed world appears to be very low, in the order of 5–10% below the age of 20[1,5–7]. Poorer countries in the developed world fall between these two patterns, as reviewed in the preceding chapter.

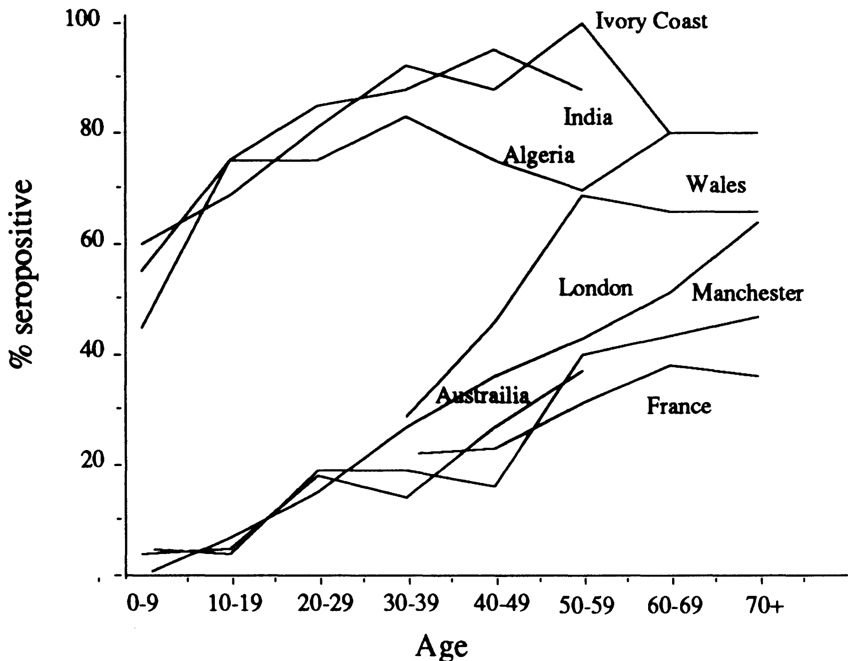

Fig. 1 The prevalence of *H. pylori* with age in the developed and developing world

DURATION OF INFECTION AND INCIDENCE

Follow-up studies of gastritis in the days before the discovery of *H. pylori* showed that it rarely regresses[8,9]. Following therapeutic eradication, gastritis disappears within 12 months[10], so that it is likely that it represents continued infection.

In the developing world the incidence is extremely high in the first few years of life, approaching 10% per annum. After 20 years of age few of the population remain uninfected. In one study from the developed world, sera were collected a mean of 12.7 years apart from a group of epidemiologists[12]; 11 of 278 subjects negative on the first occasion were positive on the second, and six of 57 subjects initially positive became negative. This supports the notion that infection once acquired is likely to be long-lived, with a spontaneous eradication rate of less than 1% per year. The incidence was in the order of 0.5% (95% CI 0.3–0.9%) per year in developed countries. There was evidence in this study of a cohort effect: subjects of a certain age at the beginning of the study had a higher prevalence of infection than subjects of the same age at the end of the study, reflecting variable exposure associated with year of birth.

An early study of incidence in children in Britain[11] also suggested a low incidence of infection (< 0.2% per annum in teenagers), but the study is difficult to interpret due to several equivocal sera.

During a total 314 patient-years of follow-up, Borody found only two

cases of reinfection following eradication treatments[13]. It is not clear whether these both occurred within the first year after treatment, in which case they may merely represent incorrectly diagnosed eradication. The situation of reinfection may be different to primary infection, where there is no pre-existing immune response.

Few follow-up studies of individuals following eradication have been performed in the developing world. Eradication is more difficult to achieve[14] owing to metronidazole resistance, but a high proportion (up to 20%) of subjects successfully eradicated become reinfected within 18 months[15]. It is not known how many of these represent incorrect diagnosis of eradication. Hence, it may be that the high rates of infection in childhood are maintained in adulthood amongst susceptible individuals. Further studies in this area are needed.

RISK FACTORS FOR ACQUISITION: CLUES TO THE MEANS OF TRANSMISSION

The best-established risk factors for *H. pylori* infection are increasing age and socioeconomic deprivation. A crowded environment has been identified as a risk factor[16], infection being more common in institutions. Prevalence of infection is increased in the families of infected children[17,18], suggesting but not proving interpersonal transmission, being also consistent with a common environmental source.

Although the prevalence of infection does differ between ethnic groups in developed countries[19], it is not clear how much of this is due to different socioeconomic circumstances. There is no relation between infection and sex, smoking, blood group[20] or alcohol consumption. Meat-eaters are at no higher risk of infection than vegetarians[21], suggesting that it is not a zoonosis. Water source has been suggested as a risk factor[22], but this has not been confirmed. Rates of infection have been shown by some[23], but not others[24], to be higher in gastroenterologists and endoscopic transmission has been reported[25].

Childhood risk factors for *H. pylori*: the importance of overcrowding and poor hygiene

In the developing world most people seem to acquire the infection early in childhood. In a cross-sectional study in the developed world, we found that adult seropositivity for *H. pylori* was strongly and independently associated with overcrowding and the absence of a fixed hot-water supply in childhood[3]. This suggests that person-to-person transmission is important, particularly as the association was independent of other measures of poverty. The association with overcrowding in childhood has since been reported by others in a developed[26] and developing country[27]. Absence of a hot-water supply is a good index of hygiene. Poor hygiene has been suggested as a risk factor for transmission, but as yet there is no other confirmatory statistical

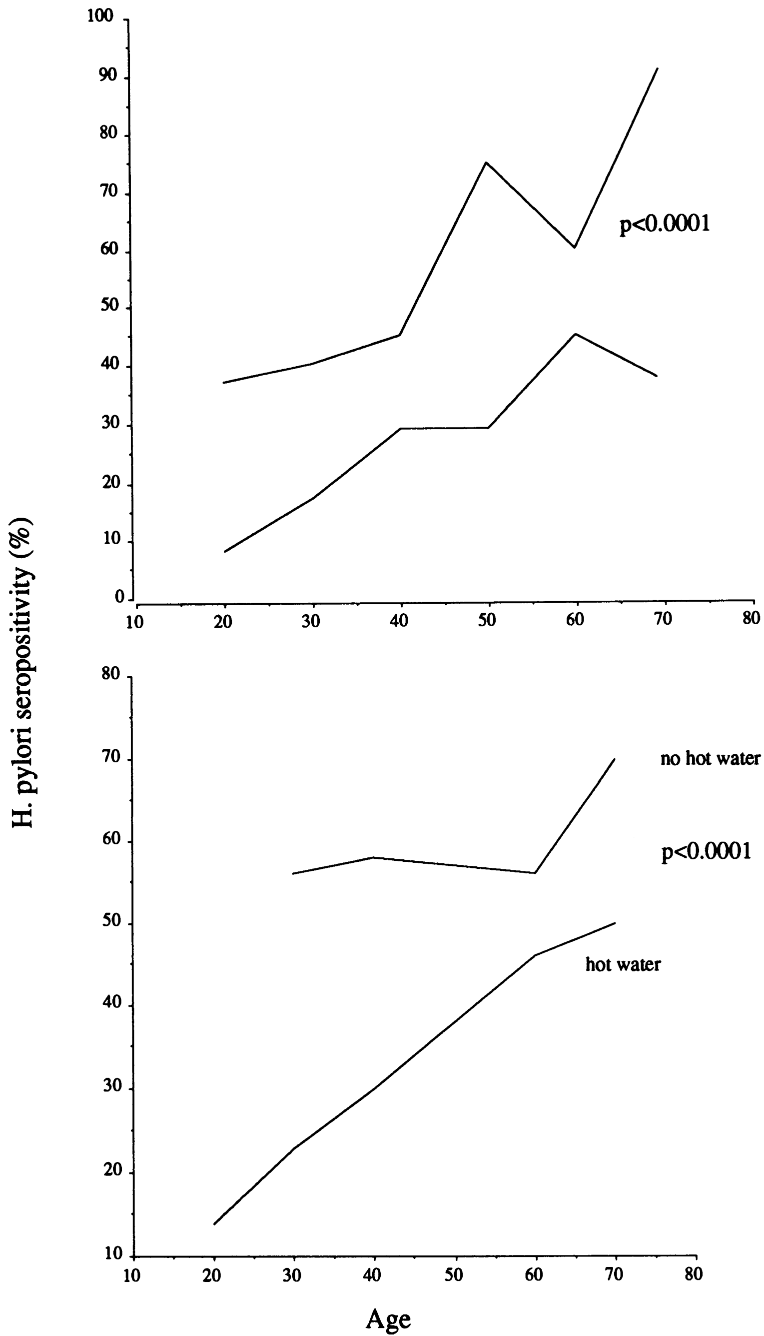

Fig. 2 The effect of childhood overcrowding and lack of a fixed hot-water supply in the childhood home on adult seropositivity among a population from London

evidence. Of interest are the high rates of infection in Japan, which is crowded, but has good levels of hygiene[28].

Adult risk factors

Risk factors for acquisition of *H. pylori* infection in adulthood are of some importance if reinfection following eradication is to be avoided. Little in this area is known in the developing world, where almost all the adult population is in any case infected from childhood.

There was no independent relation of current social class or father's social class to the presence of infection in our own study in the developed world. However, other studies have found an association between current social class and *H. pylori* seropositivity[29]. Particularly in younger subjects, social class will be heavily confounded by childhood social class. In a large population sample in Britain of 700 men 30–65 years old, social class was found to be related to *H. pylori* seropositivity only in younger subjects[4]. Whilst social class cannot be ruled out as a risk factor, the effect independent of childhood socioeconomic circumstances is likely to have been overestimated in the developed world.

The importance of number of people living in the home for risk of adult infection

If infection is interpersonal, then the two groups of people from whom adults are most likely to acquire it are their children or spouses.

In our own study[3], which we have now enlarged to 450 subjects, an important independent risk factor in adult life was the number of children currently living in the household, with an odds ratio per additional child of 1.34. This relation was independent of childhood and current living circumstances, and in particular was independent of marital status, which was of borderline statistical significance, with an odds ratio of 2.4 (0.9–6.2), owing to the small number of unmarried subjects in the study.

Evidence that transmission from children to adults may occur comes from a case report of an infant with acute *H. pylori* infection. Serologically, the patient's mother was shown to have an established *H. pylori* infection prior to her son, his twin brother was shown to have acquired the infection shortly before the patient, and his father to have become infected some 63 days after the patient[30].

We determined the *H. pylori* status of the children of seropositive adults identified in our own study where the number of children was found to be a risk factor for infection, to investigate whether they could have been the source of their parents' infection[7]. Only one of 26 children (mean age 12) of 10 seropositive mothers and one seropositive father was positive by ^{13}C urea breath test. This suggests that, among these infected adults, their children are not the source of their infection. The number of children in the household may be a marker for some other exposure, although it is also possible that

children have the ability to spontaneously clear the infection.

There are a number of reports of spouses having the same strain of *H. pylori* as each other[31,32], both by ribotyping and DNA hybridization. Strain analysis of *H. pylori* by these techniques yields a high degree of variability, with family members being the only situation in which identical strains may be isolated from different subjects. Even in this situation, most family members have different strains. If transmission is interpersonal, then spouses would seem to be capable of transmitting it to each other.

Studies of the frequency of infection in spouses of index-positive adults have given conflicting results. In a study of 277 infertile couples, no evidence was found of an increased rate of infection in partners after controlling for age and socioeconomic factors[33]. Two studies from England also gave the same conclusions. One study from America suggested that there was an increased rate of infection among the spouses of index-positive adults[34]. None of these other studies was properly controlled for socioeconomic factors, or was of sufficient size. In a population with a 30% prevalence of infection amongst adults it would take a study of 150 married couples to have sufficient power to detect an effect which led to 20% of those infected acquiring *H. pylori* from this source.

Thus, most adults with *H. pylori* infection in the developed world probably acquired it during childhood. However, as living conditions improve and more people reach adulthood uninfected, adulthood is probably becoming a more important period of acquisition. The main adult risk factors are poorly defined and much weaker than childhood risk factors, but seem to relate to contact with other people, particularly children, which is greater in young adulthood.

MODE OF TRANSMISSION

Evidence for an environmental reservoir

H. pylori has never been cultured from the environment, although this may be possible if greater efforts are made. It is able to survive for several days in distilled water, saline, and sea water if these are kept cool, but becomes non-culturable after 1–3 days at room temperature[35]. In another report *H. pylori* could be cultured from milk after several days if this was kept at room temperature.

A study from Peru[22] using the ^{13}C urea breath test showed a relation between water supply and risk of *H. pylori* infection in children. Children of high-income families who used the municipal water supply had a higher rate (37%) of infection than children from rich families who used personal wells (4%). The possibility that contaminated water may be important in the transmission of *H. pylori* has not been confirmed in other studies. The authors were unable to culture *H. pylori* from filtrates of the municipal water, which was heavily contaminated with other organisms. They suggest that it may be present in the water supply only intermittently, although water supply could be a marker of some other exposure which predisposes to *H. pylori* infection.

Oro-oral and faeco-oral transmission

For *H. pylori* to infect other subjects, assuming that humans are the only reservoir for infection, it must leave its protected environment in the gastric mucus and would be expected to be found in gastric juice, saliva, dental plaque, or faeces, depending on the route of transmission.

One of the problems with identifying *H. pylori* in human secretions away from the stomach centres on the difficulty of culture, particularly in environments such as the faeces which are heavily contaminated with other organisms which grow much faster in a microaerophilic atmosphere. One approach to this problem has been to use the polymerase chain reaction (PCR), which is a very sensitive way of detecting *H. pylori* DNA when the organism cannot be cultured. This has the drawback of also detecting non-viable organisms which are not infectious, although it is possible that organisms could exist in viable coccoidal forms which are not culturable *in vitro*. There are also theoretical problems with the specificity of the technique.

H. pylori culture from gastric juice is possible, but is less successful than from gastric biopsies, and survival may be aided by mucus and cellular debris which are aspirated from the stomach when the juice is sampled[36]. It has also been identified histologically in association with Barret's oesophagus[37] and from areas of heterotopic gastric tissue in the proximal oesophagus[38] and rectum[39]. It has been identified histologically in Meckel's diverticulum[40,41]. It has never been cultured from saliva, but has been identified by PCR.

H. pylori has been cultured from the dental plaque of dyspeptic subjects in three studies[42,43]. In one of the studies the organism was grown only in one out of 37 patients[42]. In the second study[44], *H. pylori* was cultured again from only one patient, but in the third from India[43] it was grown in 40/40 asymptomatic volunteers. Others have had difficulty culturing it from this location. Recent studies using PCR have been able to detect the presence of *H. pylori* DNA in dental plaque of dyspeptic children, and in several cases it was not recoverable from the stomach. It is possible that dental plaque is acting as a reservoir of infection, with the organism only being able to colonize the stomach when conditions are favourable. Whether or not *H. pylori* in an infectious form is frequently present in dental plaque, it is feasible that the organism is present transiently in the mouth from contamination by gastric juice during episodes of oesophageal reflux.

Earlier attempts to culture *H. pylori* from faeces were unsuccessful. However, *H. pylori* DNA has been detected using PCR[45], and has been cultured from the faeces of African children[46]. Given the organism's ability to survive for at least a limited time outside the human body, this suggests faeco-oral transmission as a possibility.

Animal experiments with the mouse which can be experimentally infected with a similar organism to *H. pylori*, *H. felis* (natural host the cat), have shown failure of mice to transmit the infection to other non-infected mice. This is of interest, because mice are coprophagic, suggesting that transmission by the faeco-oral route does not occur. On the other hand, germ-free puppies did transmit *H. pylori* to each other. These animals have frequent oro-oral contact, suggesting that oro-oral transmission can occur[47].

Parallels with hepatitis A (faeco-orally transmitted) and infectious mononucleosis (oro-orally transmitted)

Once infected with either of these viruses, there is a lifelong immune response, so that they may be good models for studying the mode of transmission of *H. pylori.*

In the developing world both infections are acquired at an early age with virtually the whole population infected by the age of 5. However, in the developed world the pattern of seroprevalence differs. In hepatitis A there is an age cohort effect with a gradual rise in prevalence with age, reflecting the fact that the period of most risk is in early childhood when faeco-oral transmission most commonly occurs. Infectious mononucleosis, however, shows two main periods of acquisition. Again there is an important period of acquisition in early childhood when oro-oral contact is common. Then from the age of 5–15 the rate of acquisition is low, but becomes greatest of all between the ages of 15 and 30, leaving the whole population infected. This is due to frequent oro-oral contact with other people during adolescence and early adulthood. This does not appear to be a major period of acquisition of *H. pylori* in the developed world, but we found that the association with marital status was more marked in young adults, suggesting that oro-oral transmission may be important in this age group.

Thus, the epidemiology of *H. pylori* parallels faeco-oral transmission, but to date it has been easier to isolate the organism from the mouth than the faeces. This is probably due to technical reasons, rather than an absence of the organism in this location. However, it is likely that transmission occurs by both routes, the precise route depending on the age and circumstances. For example, transmission between young adults in the developed world is likely to be oro-oral, whilst for children in the developing world the faeco-oral route may be more important. Further attempts to identify the organism in the environment are required.

HOST FACTORS AND THE TRANSMISSION OF *H. PYLORI*

H. pylori is unable to survive for long in an acid environment, but it can survive longer than other organisms particularly in the presence of urea[48]. In the volunteer ingestion study by Marshall[49], the infecting dose was taken after premedication with cimetidine. Morris attempted to infect himself first with a dose of 4×10^7 colony-forming units without premedication with cimetidine using the same strain as that used by Marshall, and failed. However, he succeeded using a smaller infecting dose of 3×10^5 CFU after premedication with cimetidine to render the gastric pH neutral[50].

Ferrets are infected by an organism, *H. mustelae*, which is associated with many of the pathological features seen with *H. pylori* in humans. The physiology of the ferret stomach is very like that of the human stomach[51]. Forty-one per cent of adult ferrets given omeprazole to render the gastric contents neutral had positive faecal cultures as opposed to 10% of ferrets not given the drug[52].

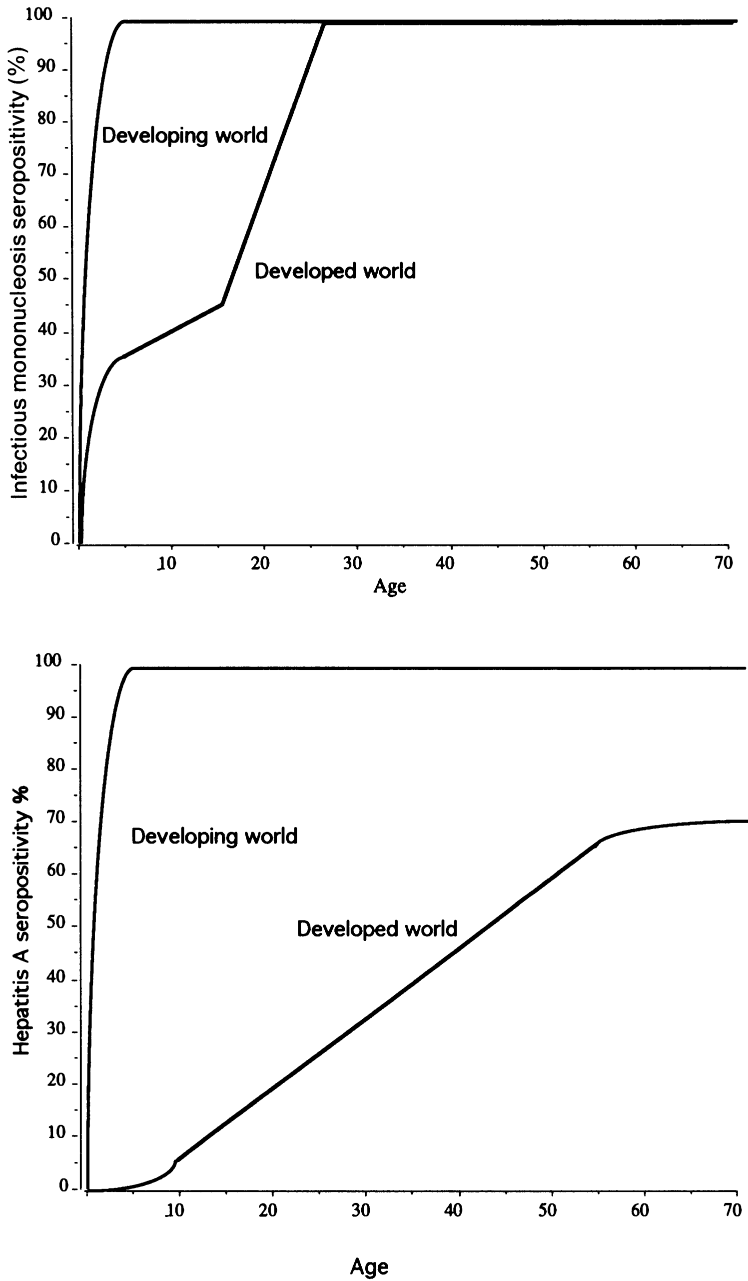

Fig. 3 Seroprevalence with age of hepatitis A, a faeco-orally transmitted infection and infectious mononucleosis, an oro-orally transmitted infection

A variety of bacterial and parasitic infections have been reported to suppress gastric acid secretion in humans, including typhoid, paratyphoid, pulmonary tuberculosis, bronchopneumonia, lung abscess, Chagas' disease, fish tapeworm, *Giardia* and hookworm infestations[53,54]. The mechanism involved in this reduction of gastric acid secretion in infection is unclear, but the induction of short-term pyrexia in normal volunteers also suppresses gastric acid secretion, in some cases completely[55]. Malnutrition is also a recognized cause of reduced gastric acid secretion[54].

Bile acids are toxic to *H. pylori*, and could limit its transmission down the intestine. Bile acid concentrations are reduced in malnutrition and in diarrhoeal diseases, suggesting further how infectious disease and malnutrition may facilitate transmission.

As a result of these factors, one would expect transmission to be more common in the developing world and in children. This would also explain the low infectivity in the developed world by analogy with tuberculosis, whose decline in transmission was due in part to decreased host susceptibility. An interesting observation is that in studies, including our own, in which height has been determined, *H. pylori*-infected individuals are shorter[22,56]. This may not be totally explained by socioeconomic factors, and no good evidence points to it being a consequence of infection.

CONCLUSION

Much work still needs to be done on the epidemiology of *H. pylori* infection. In most cases infection, once acquired, is for life. In the developed world, as in the developing world, infection is mainly acquired in childhood, but significant acquisition also probably occurs in young adulthood. Faeco-oral or oro-oral transmission may occur, and evidence suggests that this may be facilitated by hypochlorhydria, perhaps induced by malnutrition and a wide range of other infections. *H. pylori* has yet to be identified in the environment, but is probably present as a contaminant.

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PATHOPHYSIOLOGY

4
Pathogenesis of infections due to persistent bacteria at mucosal surfaces

MARTIN BLASER

INTRODUCTION

Helicobacter pylori and *Campylobacter fetus* are two spiral Gram-negative bacteria that have evolved lifestyles centred on long-term persistence in the mucus gel overlapping epithelial tissues. Since the discovery of *H. pylori* in 1983 there has been intense interest in this organism because of its relationship to ulcer disease, among other maladies. However, the pathophysiology of *H. pylori* infection is largely unknown. In this chapter I review the major points that have been learned about *H. pylori* pathogenesis, and then review what is known about the pathogenesis of *C. fetus* infection. It is hoped that bringing together information about these two different organisms that contend with parallel constraints can provide new insights.

NATURAL HISTORY OF *H. PYLORI* INFECTION

H. pylori infection is highly associated with the presence of an inflammatory condition that is called chronic superficial gastritis (also known as active chronic gastritis)[1,2]. Essentially all persons who become infected with *H. pylori* develop chronic superficial gastritis involving the antrum and the fundus, whether or not they have gastrointestinal symptoms[3]. The inflammatory infiltrate consists of mononuclear cells and usually polymorphonuclear leucocytes, and epithelial cell structure and function are impaired. The evidence now is clear that *H. pylori* causes this tissue injury[2].

Although our knowledge of the natural history of *H. pylori*-induced chronic superficial gastritis is as yet incomplete, several points now are relatively certain. In most infected persons, chronic superficial gastritis persists for essentially the lifetime of the host[4,5]. In others, however, the inflammatory process is associated with permanent flattening or loss of epithelial glands,

termed atrophic gastritis[6]. Data from long-term longitudinal studies conducted in Scandinavia indicate that chronic superficial gastritis may progress first to moderate and then to severe atrophic gastritis over a period of decades[7]. In the Scandinavian studies the average interval between chronic superficial gastritis and moderate or severe atrophic gastritis was greater than 37 years. Atrophic gastritis is of particular importance because it is well recognized as a risk factor for gastric cancer[8]. Recent studies have also shown that *H. pylori* infection is a strong risk factor for the development of gastric cancer[9–11]. Another important outcome of *H. pylori* infection is the development of peptic ulcer disease[4,12]. Bacterial-induced inflammation alters the normal somatostatin–gastrin–hydrochloric acid–pH homeostasis, a phenomenon that may contribute to an ulcer diathesis[13].

However, in most persons, *H. pylori* is clinically silent; adverse consequences, such as peptic ulcer disease and adenocarcinoma of the stomach, occur only in a minority of infected persons. Thus, two questions are of major importance in understanding the pathogenesis of this infection. First, it is necessary to understand the mechanisms responsible for *H. pylori*-induced chronic inflammation; and second, the factors determining the heterogeneity of outcomes must be elucidated.

MECHANISMS OF INFLAMMATION

For understanding how inflammation occurs, the central question is whether or not *H. pylori* invades the gastric mucosa. Most *H. pylori* organisms are free-living in the mucus gel that covers the epithelium[14]. A small ($< 5\%$) fraction of organisms adhere to epithelial cells[15,16]. It is now clear that *H. pylori* does not invade beyond the epithelium, and if there is actual invasion of epithelial cells this phenomenon occurs to a minimal degree. Thus, mucosal injury must be due to released bacterial products or to the effects of bacterial proliferation on homeostatic mechanisms for viability of the mucosa[17–19]. Current efforts are focused on the first possibility, and a number of putative virulence factors including lipopolysaccharide, cytotoxins, urease, and ammonia are being actively studied[20–25]. A local and systemic antibody response to *H. pylori* indicates that the host recognizes the presence of the organism, but the specific mechanisms in the different responses have not been well characterized. It is possible, perhaps likely, that the host inflammatory response to *H. pylori* contributes to both tissue damage and to disordered gastric secretory physiology.

FACTORS DETERMINING HETEROGENEITY OF OUTCOME OF *H. PYLORI* INFECTION

For understanding the diversity of outcomes of *H. pylori* infection, four types of factors should be considered. First, there may be heterogeneity of *H. pylori* strains. Although most phenotypic characteristics are well conserved, we are now identifying properties (such as the *cagA* gene product — a 128 kDa

protein) that are not present on all strains[26]. Second, there may be heterogeneity in host responses. As with other infectious diseases, genetic factors determining immune response (Ir genes) could influence response to this chronic infection. Third, the age at which *H. pylori* is acquired may have a bearing on infection outcome as it does for many infectious diseases (including varicella, hepatitis A, and Epstein–Barr virus infections, for example). Fourth, there may be environmental co-factors that interact with the tissue response to *H. pylori* infection. Although *H. pylori* infection is highly prevalent throughout developing countries, the incidence of gastric cancer varies greatly. Epidemiological studies have implicated a number of other environmental risk factors for gastric cancer[27] that might potentiate the pathogenetic role of *H. pylori*. Similarly, smoking appears to be an independent risk factor for peptic ulceration. The relative contributions of these four types of factors are unknown at present; this will surely be an important field of inquiry.

CAMPYLOBACTER FETUS INFECTION AS A MODEL FOR *HELICOBACTER PYLORI* INFECTION

Campylobacter fetus subsp. *venerealis* is a pathogen of cattle that causes infertility and enzootic abortion[28]. The lifestyle of this extracellular organism is centred around chronic carriage (lasting for years) on mucosal surfaces of infected animals. For both *H. pylori* and *C. fetus*, a mammalian host appears obligatory. Since much has been learned over the past 80 years about the pathogenesis and immunology of *C. fetus* infections of cattle (and sheep), it may be useful as a model for developing strategies to prevent and treat *H. pylori* infections.

THE ORGANISM

Like *H. pylori*, *C. fetus* are microaerophilic, spiral, motile, curved Gram-negative rods[28]. *C. fetus* may multiply between 25°C and 37°C, a range that reflects its particular environmental niches. Its lipopolysaccharide (LPS) is characterized by long polysaccharide side-chains of variable length[29]; for subsp. *venerealis* there is only a single serotype (A)[30]. The major virulence feature of these organisms is the presence of a regular paracrystalline surface (S-)layer, composed of acidic high molecular weight proteins[31–33]. As has been learned from the closely related organism, *C. fetus* susbp. *fetus*, these S-layer proteins have molecular weights ranging from 85 to 149 kDa, but only a single protein predominates on each strain[33–35]. The presence of the S-layer renders *C. fetus* resistant to serum-killing and phagocytosis, based on the inability of C3b to bind to these cells[36,37]. This is an important property for an extracellular organism that infects mammals, but infected hosts are able to produce specific antibodies to *C. fetus* determinants that are opsonic and that permit phagocytosis via Fc receptors[37,38]. However, *C. fetus* has the ability to change the major S-layer protein on its sur-

face[35,39,40]. This switch is associated with presentation of new antigens to the host[41]. Thus, the S-layer protein is extremely useful to *C. fetus*, thwarting both antibody and complement[31,42,43]. This property is likely to be responsible for the long-term carrier state associated with natural *C. fetus* infections.

In comparison, infected hosts develop both local and systemic antibodies directed against *H. pylori*[44,45], and these organisms are susceptible to serum-mediated killing *in vitro*[46]. However, *in vivo*, both complement and antibody also are apparently ineffective against *H. pylori*; presumably this is due to the location of the organism in the gastric milieu, where these host defences cannot work efficiently.

C. FETUS IN CATTLE

In the bull, *C. fetus* subsp. *venerealis* lives in the penile prepuce. More specifically, these organisms reside in the mucus layer overlaying the preputial epithelium[47], and do not appear to adhere to or invade the epithelium. The host recognizes that the organisms are present, as demonstrated by the observation that preputial fluids contain specific IgA and IgG[48]. However, the organisms may persist in this site for years[47,48]. *C. fetus* subsp. *venerealis* is transmitted to a cow during sexual intercourse with an infected bull[38,47]. The organism colonizes the endometrium, and the host develops an inflammatory response composed of both polymorphonuclear and mononuclear cells. This inflammatory process resembles that induced by *H. pylori*. As with *H. pylori*-induced inflammation, this process also has clinical consequences in that an inflamed endometrium is not a suitable site for implantation of fertilized ova, and the cow becomes infertile. Infected cows develop a local antibody response, and after several months can eliminate the organism from the endometrium, and fertility (i.e. normal function for that mucosal epithelium) is restored[38]. However, colonization of the vagina can persist for years despite the presence of secretory IgA directed against the organism, presumably because of the antigenic variation of the S-layer. Again, as with *H. pylori*, this organism is able to persist despite the presence of specific local antibodies. Parenteral vaccination of either bulls or cows with formalin-killed whole *C. fetus* cells induces specific serum antibodies, and these animals are protected from infection[49]. Of even greater interest is that parenteral vaccination can eliminate *C. fetus* in chronically infected cows and bulls[50,51]. The mechanisms for these phenomena are not well understood.

LESSONS

As with *H. pylori*, *C. fetus* is superbly adapted for persistence on mucosal surfaces of infected hosts[52,53]. Presumably, evolutionary pressures over the millennia have selected for strains that are sufficiently well adapted to enable this long-term colonization, but not so virulent as to destroy its environmental

niches. However, its evolution had not prepared *C. fetus* for human intervention, in that there had been no selective pressure to thwart the effects that parenteral challenge and antigen presentation might have on mucosal colonization. By analogy, it is possible that chronic mucosal infections such as *H. pylori* may be prevented or cured by interventions for which its evolution has not prepared it, such as parenteral challenge[54]. This is a hypothesis worth testing in a suitable animal model.

CONCLUSIONS

H. pylori is exquisitely well adapted for long-term carriage by human hosts. As with other pathogenic bacteria, the 'goal' of these organisms is to stably infect a host before being transmitted to a new host. It is not certain whether we should distinguish between bacterial properties that favour long-term colonization, and properties that we arbitrarily consider as 'virulence factors'. Pathogenesis is undoubtedly a complex process centred on the persistent colonization of *H. pylori* at a site that is normally free of bacteria. Mechanisms that the host uses to limit the inflammation induced by *H. pylori* may be important in the maintenance of the ecologic niche for the organism, and these are possibly tightly regulated. Study of other persistent mucosal colonizers, such as *C. fetus*, may be useful in understanding the pathogenesis of *H. pylori* infections.

In addition to the importance of *H. pylori* in gastroduodenal disease, understanding the mechanisms by which this infection causes tissue injury may aid in understanding the pathogenesis of other chronic mucosal infections, as well as chronic inflammatory conditions of mucosal surfaces that are presently of unknown aetiology. This is a rich area for scientific inquiry.

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5
Host responses

JEAN CRABTREE and JANE WYATT

INTRODUCTION

It is now clear that *Helicobacter pylori*, one of the commonest bacterial infections of man, is the causative agent of chronic gastritis. Volunteer, treatment and animal model studies all implicate this organism as the aetiological agent inducing gastric inflammation. Many infected subjects remain asymptomatic. However, the increasing importance of *H. pylori* in peptic ulcer disease[1], gastric adenocarcinoma (Chapter 2 of this volume) and gastric MALT lymphoma[2,3] stresses the relevance of this bacterial infection to gastroduodenal disease. Whilst several potential bacterial pathogenic factors have been described in the chapter by Blaser (Chapter 4) in this volume, our understanding of the role of *H. pylori* in the pathogenesis of gastroduodenal disease is still limited. The local immune response to the organism, characterized by infiltration of plasma cells, neutrophils, lymphocytes and monocytes, may be an important factor in the pathogenesis of *H. pylori*-associated gastroduodenal lesions. Variations in host responses to infection determined by immune response genes may be critical in determining the extent of *H. pylori*-induced mucosal injury. There is also increasing evidence that inflammatory responses may reflect strain variation in *H. pylori*[4,5].

HUMORAL RESPONSES

Chronic gastritis is characterized by an increase in mucosal plasma cell density and increased epithelial expression of secretory component[6,7]. Early studies, following the identification of *H. pylori* as the aetiological agent of chronic gastritis, demonstrated that infection was associated with a specific gastric IgG and IgA response to the bacterium[8]. A specific IgA and IgG response to *H. pylori* also occurs in the duodenal bulb mucosa of patients with duodenitis[9]. The local duodenal antibody response is evident only in the first part of the duodenum, the site of *H. pylori* colonization of gastric metaplasia[10], as no specific *H. pylori* IgA antibodies are secreted by biopsies

from the second part of the duodenum in subjects with duodenitis of the bulb mucosa. There is little evidence of a specific mucosal IgM response to the bacterium except in acute gastritis[11].

Characterization of the antigen specificity of the antral IgA response to *H. pylori* by immunoblotting in patients with chronic gastritis demonstrated the marked heterogeneity in antigen recognition patterns[4] (Fig. 1). Within the group of patients with chronic gastritis an IgA response to a 120 kDa protein was strongly correlated with peptic ulcer disease, the activity of gastritis (i.e. epithelial neutrophil infiltration) (Fig. 2A) and the extent of epithelial surface degeneration (Fig. 2B), but was independent of bacterial density assessed histopathologically (Fig. 2C). The relationship between 120 kDa antigen recognition in chronic gastritis and polymorph infiltration was also apparent when only patients with non-ulcer dyspepsia were examined (Fig. 3). As not all strains of *H. pylori* express this 120–130 kDa cytotoxin-associated protein[12,13], the variations in local antibody response are likely to reflect the bacterial strain with which the host is infected. Whether the association between the severity of gastritis and mucosal IgA recognition of the 120 kDa protein relates directly to a function of this protein, the related 87 kDa cytotoxin[14] or other associated factors is currently unclear. As gastric mucosal culture supernatants containing *H. pylori*-specific IgA antibodies will inhibit *in vitro* cytotoxicity[15], other bacterial factors associated with the cytotoxin may be more important in ulceration and the development of active gastritis.

Serologically, patients with duodenal ulcers also have a high frequency of IgG recognition of the 120–130 kDa protein[16,17]. An ELISA based on a purified recombinant fragment of the 128 kDa protein[13] has confirmed these Western blot observations[18]. Increased systemic IgG recognition of the 120 kDa protein is also evident in patients with gastric cancer[19]. A mucosal and systemic humoral response to a protein only expressed in certain strains therefore occurs in *H. pylori*-infected patients who develop serious gastroduodenal lesions. To date no specific function has been attributed to the 120–130 kDa protein.

Whilst *H. pylori* infection is associated with a strong mucosal antibody response against several bacterial components, crossreacting epitopes between *H. pylori* and the gastric mucosa have also been demonstrated[20]. *H. pylori* monoclonal antibodies have been shown to crossreact with both human and murine gastric epithelia[20] and patterns of reactivity suggest at least three crossreacting epitopes[21]. In infected subjects, serum autoantibodies to the antral mucosa are present and preabsorption with *H. pylori* abolishes reactivity[20]. The crossreactivity may reflect recognition of a recently cloned 58 kDa protein of *H. pylori* which belongs to the heat-shock protein family hsp60[22]. This 58 kDa urease-associated protein, which has a high degree of homology with human hsp60, is recognized serologically in most patients with *H. pylori* infection[22]. Autoimmune responses against heat-shock proteins or other crossreacting host–bacterial epitopes may have a role in the pathogenesis of gastritis. Autoantibody responses generated by *H. pylori* infection also include a local IgA response to the neutrophil activating and chemotactic cytokine interleukin-8 (IL-8)[23]. Whether these IL-8 autoantibod-

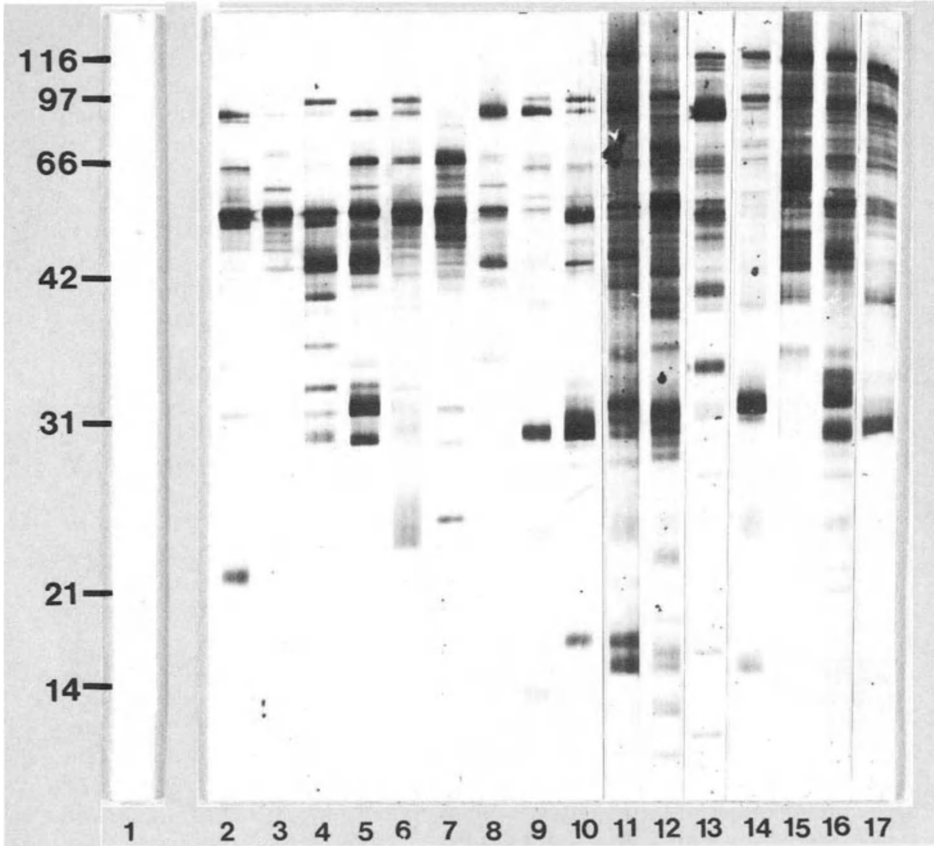

Fig. 1 Western blot of IgA antibodies in antral biopsy culture supernatants to whole cell preparations of *H. pylori* (NCTC 11637). Track 1 = control with normal gastric mucosa. Tracks 2–17 = patients with chronic gastritis. Tracks 11–17 show recognition of the 120 kDa protein. Figures on the left are protein molecular weight standards in kDa.

ies have a role in pathogenesis of gastritis or represent a protective down-regulatory mechanism of the host to block excessive IL-8-induced neutrophil activation is currently unclear.

IgA responses at mucosal sites are thought to be functional in decreasing bacterial motility, inhibiting adherence, neutralizing biologically active bacterial products and preventing antigen uptake[24]. Additionally, IgA antibodies may also block IgG-mediated responses, complement activation and associated tissue damage. The absence of an antibody response in patients with agammaglobulinaemia has been associated with the early development of atrophic gastritis[25]. As atrophic gastritis is a risk factor for gastric cancer[26], it is interesting that subjects with hypogammaglobulinaemia have a 50-fold increased risk of developing gastric cancer[27]. Enhanced antigen uptake in

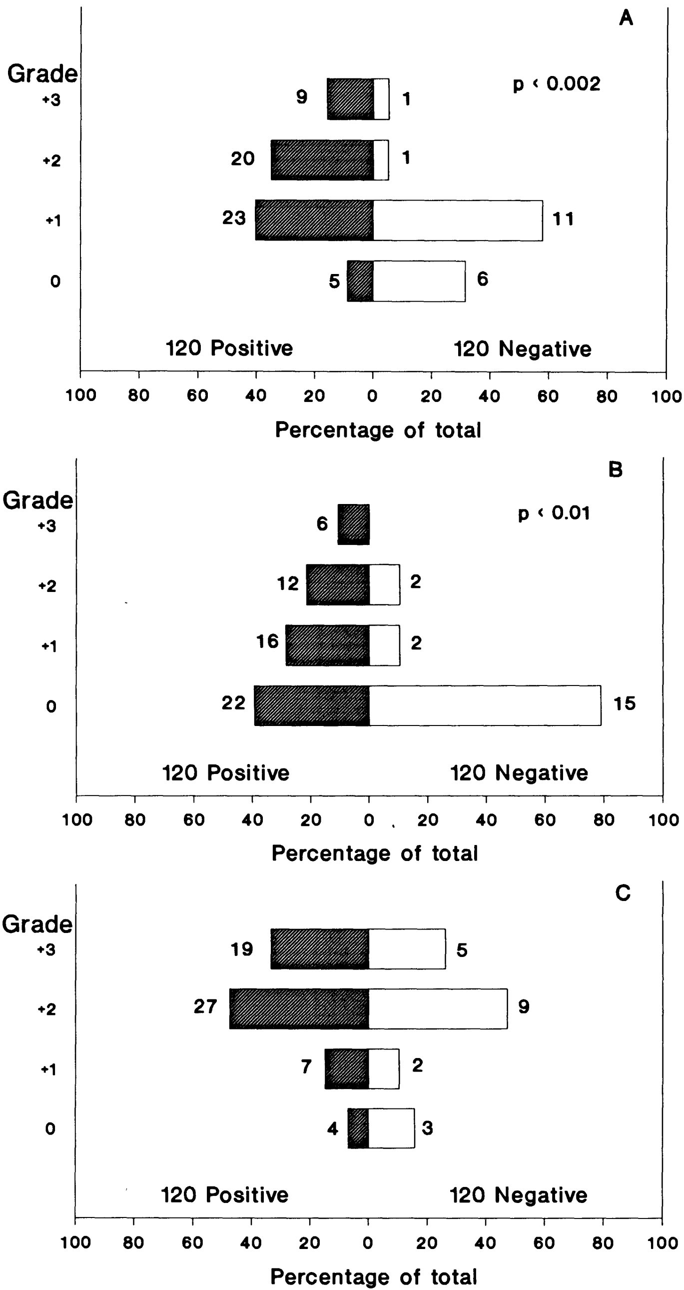

Fig. 2 Relation between polymorph density (A: $n = 76$), epithelial surface degeneration (B: $n = 75$) and *H. pylori* density assessed histopathologically (C: $n = 76$) and mucosal IgA recognition of *H. pylori* 120 kDa protein in patients with chronic gastritis. Numbers of patients are given beside blocks. Reproduced with permission of the *Lancet*

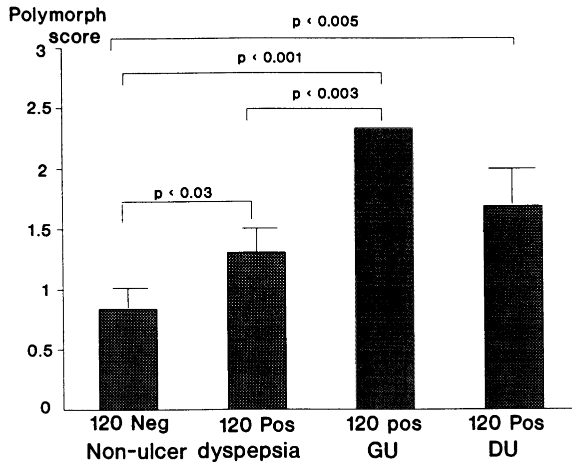

Fig. 3 Relation between activity of gastritis (polymorph score) and IgA recognition of *H. pylori* 120 kDa protein in patients with chronic gastritis according to endoscopic diagnosis ($n = 76$)

the absence of protective blocking antibodies may induce a rapid development of atrophic gastritis.

With the development of atrophic gastritis and extensive intestinal metaplasia, changes in gastric microflora associated with hypochlorhydria may militate against continued infection with *H. pylori*[28,29]. *H. pylori* serological positivity can be evident in patients with *H. pylori*-negative atrophic gastritis with[26] or without gastric cancer[28,29]. Additionally, some seronegative patients with gastric cancer have evidence of a local IgA response to *H. pylori*[19] indicative of previous infection. The kinetics of reduction in systemic *H. pylori* IgG antibodies following loss of infection in atrophic gastritis, have not been established. The pattern may well differ from that following therapeutic eradication.

Despite the strong local antibody response to *H. pylori*, infection is generally chronic. Immunohistological studies have shown antibody coating of bacteria *in situ*[30], but whether this represents antigen-specific recognition or Fc receptor binding is currently unclear. *In vitro* studies have shown that IgG antibodies to *H. pylori* will promote complement-dependent phagocytosis and killing of *H. pylori* by polymorphs[31]. Such studies, however, have not been undertaken in the presence of physiological levels of urea. Apart from inhibiting acidification of lysosomal compartments of neutrophils[31], ammonium will also inhibit a range of neutrophil functions by altering cytoskeletal actin[32]. The local production of ammonia following hydrolysis of urea by the urease enzyme may impair the functional capacity of mucosal neutrophils.

T-CELL RESPONSES

There have not been extensive studies of gastric T cells. In common with other intestinal sites, the gastric $CD4^+$ (helper–inducer) subset is composed almost exclusively of antigen-committed ($CD45RO^+$) cells[33]. However, there is some evidence that the gastric intraepithelial T cell population, which are mainly of the $CD8^+$ (cytotoxic/suppressor) subset, show increased expression of CD45RO in gastritis[34]. It has been suggested that T cells of the variant TcR $\gamma\delta^+$ type are increased in the epithelial compartment in gastritis, in association with expression of *groEL* heat shock protein[35]. However, careful enumeration of $CD3^+$ T cells co-expressing TcR $\gamma\delta$ in a variety of enteropathies has not shown any increase in the percentage of TcR $\gamma\delta^+$ cells in gastritis[36].

A proportion of patients with *H. pylori* infection develop lymphoid follicles in the gastric mucosa. Mucosal lymphoid follicles were observed in 27–54% of patients with *H. pylori*-associated gastritis[2,37] and their presence is dependent on continued *H. pylori* infection[3]. In gastric MALT lymphoma the frequency of *H. pylori* infection is extremely high (92%)[2]. Recent studies have shown the immunoglobulin specificity of three low-grade primary B cell gastric MALT lymphomas was for autoantigens[38]. *H. pylori*, however, did stimulate proliferation of these B cell lymphomas *in vitro* in a T-cell-dependent manner[39]. The *H. pylori*-induced proliferation of gastric B cell lymphoma cells was specific to a single different strain in each patient. A specific mucosal T cell response to *H. pylori* would therefore appear to augment expansion of autoreactive low-grade gastric B cell lymphomas[39].

H. pylori will induce peripheral blood lymphocyte proliferation in both seropositive and seronegative subjects[40], with the latter being more responsive. As *H. pylori* also induces a strong proliferative response in cord blood lymphocytes, up-regulating CD25 expression on both CD4 and CD8 T cell subsets[41], a non-specific activation mechanism may be involved. However, the reduced proliferative responses in seropositive subjects could also reflect sequestration of responsive cells to the gastrointestinal mucosa. The development of antigen-specific T cell clones from the gastric mucosa and characterization of their functional properties and cytokine secretion profiles should facilitate an understanding of the role of mucosal T cells in the immunopathology of chronic gastritis.

CYTOKINES AND CELLULAR RESPONSES

A feature of chronic gastritis is the increased expression of MHC class II antigens on the gastric epithelium, which is closely related to the density of mucosal T cells[34,42]. Enhanced MHC class II expression is thought to be under cytokine regulation, being induced by interferon γ and subject to further enhancement by cytokines such as TNF-α. Although ICAM-1 ('intercellular adhesion molecule' 1) is up-regulated in most cell types by TNF-α, there is no evidence that ICAM-1 expression can be induced or up-regulated in gastrointestinal epithelial cells. No detectable ICAM-1 expression

has been observed by mucosal epithelial cells in gastritis[43], coeliac disease[44] and inflammatory bowel disease[45], despite the massive up-regulation by lamina propria cells in the latter[45]. These observations suggest that gastrointestinal epithelial cells *in situ* do not act as antigen-presenting cells to mucosal T cells via the 'classic' pathway of antigen recognition by the T cell receptor, which depends on MHC antigen recognition and the LFA-1/ICAM-1 interaction[33]. Up-regulation of epithelial MHC class II expression may therefore be coincidental, rather than of central functional significance.

H. pylori has been shown to activate peripheral blood monocytes or polymorphs in a variety of ways. Surface proteins, lipopolysaccharide (LPS) and whole bacteria will induce TNF-α and IL-1β message expression and protein secretion in blood monocytes[46]. Up-regulation of monocyte expression of MHC class II and the IL-2 receptor and secretion of the reactive oxygen intermediate superoxide anion are also induced by the above three bacterial preparations[46]. Additionally, both peripheral blood monocytes and neutrophils produce reactive oxygen metabolites on exposure to *H. pylori*[47]. Enhanced mucosal reactive oxygen metabolite production has been observed in patients with duodenitis and duodenal ulceration, suggesting that activation of mucosal cell populations occurs in *H. pylori* infection[48]. The mucosal production of reactive oxygen metabolites may have a detrimental effect on the viscoelastic properties of gastric mucus[49]. Additionally, they may increase cytokine gene expression by activating NF-kappaB transcription factor[50].

H. pylori infection is particularly characterized by a neutrophilic response. These cells will secrete not only reactive oxygen metabolites but a range of proteolytic enzymes[51] which may be relevant to tissue damage and ulceration. Whilst several *H. pylori* neutrophil chemotactic factors have now been described[52,53], mucosal cytokines generated by infection, particularly IL-8[23], a potent neutrophil chemotactic and activating factor, may also regulate neutrophil migration and activation. Antral mucosal production of the proinflammatory cytokines TNF-α, IL-6[54] and IL-8[23] are increased in *H. pylori* infection, with TNF-α and IL-8 being specifically elevated in active gastritis (i.e. intraepithelial neutrophil infiltration). TNF-α, whilst not directly chemotactic for neutrophils, will induce expression of IL-8 in a range of cell types[55].

Gastric epithelial cell lines have recently been shown to constitutively express IL-8 mRNA, and immunoreactive IL-8 is evident in the epithelium of both normal and gastritic mucosa (Fig. 4), with expression being increased in the latter[56]. Epithelial IL-8 is also present in gastric adenocarcinoma (Fig. 5), demonstrating that expression of this cytokine is not dependent on luminal stimulation. IL-8 production in gastric epithelial cell lines can be up-regulated by TNF-α and IL-1α[57] and IL-1β (Crabtree unpublished observations). Gamma interferon has been shown to synergistically enhance TNF-α-induced IL-8 production in one gastric epithelial cell line through synergistic activation of transcription factors[57]. It is likely therefore that the local production of TNF-α, IL-1α and IL-β from macrophages within the lamina propria following bacterial stimulation will up-regulate epithelial IL-8 production (Fig. 6)[58] in *H. pylori* infection. Activated neutrophils will also

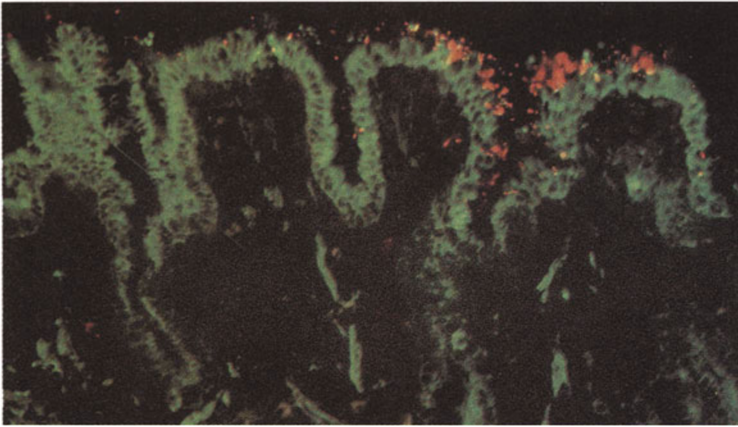

Fig. 4 Antral biopsy of patient with *H. pylori* infection and chronic gastritis double immunolabelled with a mouse monoclonal antibody to IL-8 and a rabbit antibody to recombinant urease. Second-layer antibodies were a FITC-conjugated goat anti-mouse IgG and a TRITC-conjugated goat anti-rabbit Ig. The epithelium shows strong positivity for IL-8

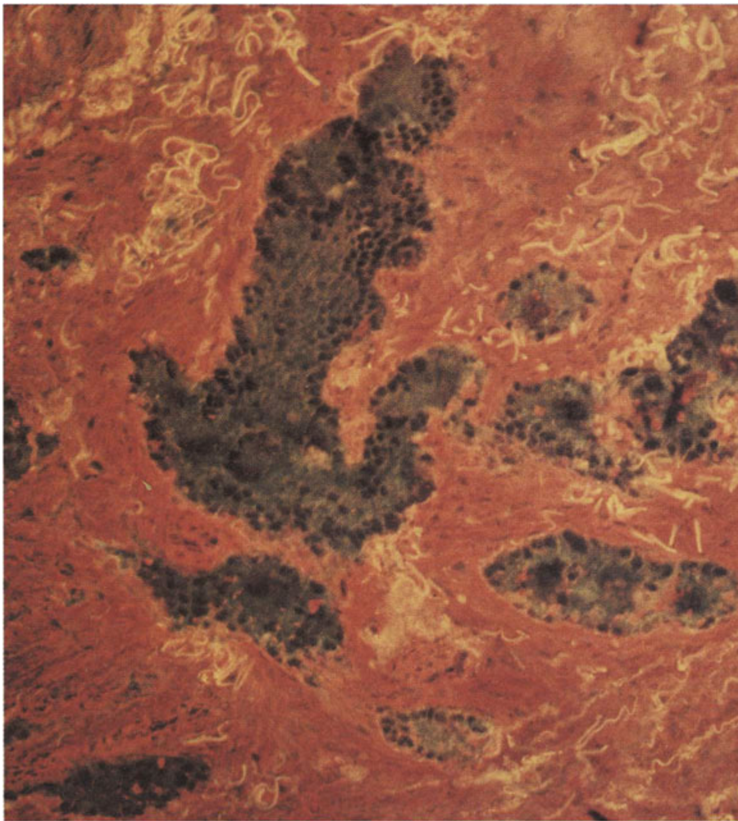

Fig. 5 Intestinal-type gastric adenocarcinoma double immunolabelled with a mouse monoclonal antibody to IL-8 and a rabbit anti-fibronectin antibody. Second-layer antibodies were a FITC-conjugated goat anti-mouse IgG and a TRITC-conjugated goat anti-rabbit Ig

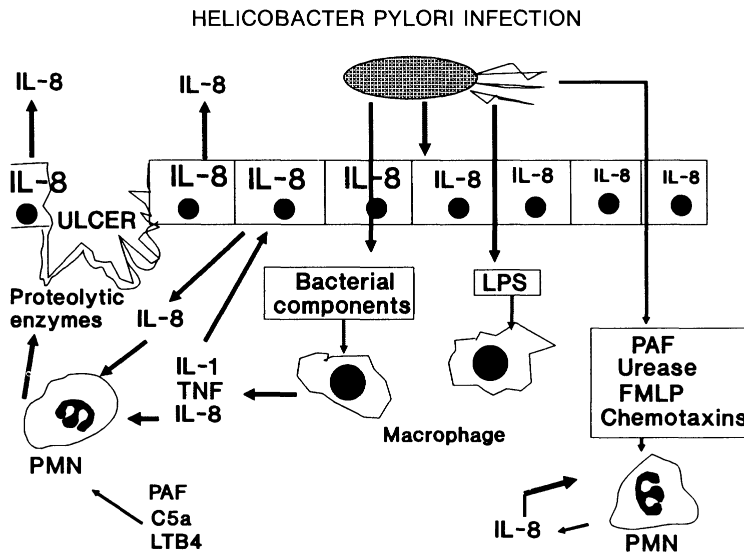

Fig. 6 Diagram showing pathways of *H. pylori* induction of epithelial IL-8 expression and possible pathogenic consequences. Reproduced with permission of the *European Journal of Gastroenterology and Hepatology*[58]

secrete IL-8, thus attracting further neutrophil infiltration[59]. *H. pylori* will also directly stimulate IL-8 secretion by gastric epithelial cell lines[60]. Cytotoxic positive strains induce significantly greater IL-8 secretion than negative strains[60], which may explain why the mucosal IgA recognition of the cytotoxin-associated 120 kDa protein is associated with more severe gastritis (i.e. epithelial neutrophil infiltration).

The ability of epithelial cells to up-regulate IL-8 expression and secretion is probably a key factor in defence of mucosal surfaces, permitting a rapid infiltration of polymorphs to eliminate infection. Clearly, with *H. pylori* infection the infiltrating neutrophils are not effective at clearing the bacteria, and continued stimulation of IL-8 and neutrophil activation could have important consequences for mucosal integrity. The development of a local IgA antibody response to IL-8 may be a down-regulatory response of the host to limit mucosal damage associated with a chronic bacterial infection[23].

Physiological responses to infection

Changes in gastrin, somatostatin and GRP-stimulated acid secretion are all associated with *H. pylori* infection (reviewed in detail in Chapter 6) and these changes are reversed following eradication of infection. It is currently unclear whether the perturbations in gastric physiology are mediated directly by *H. pylori* or other gastric microenvironmental stimuli. Mucosal cytokines may have an important role not only in regulating immune responses but in modifying aspects of gastric physiology. There is some evidence that gastrin release may be stimulated by gastric immune responses[61]. Investigations in animal models are required to determine whether bacterial or host factors

are involved in the disruption of the gastrin–acid interactions. Inflammatory mediators generated by *H. pylori* infection may also influence other non-specific mucosal defence mechanisms such as gastric mucus secretion[62].

SUMMARY

H. pylori infection offers a unique unibacterial system with which to investigate human mucosal immune responses. Infection stimulates a marked mucosal humoral and cellular response, which in part may be influenced by strain differences of colonizing bacteria. Despite the strong host response, infection is chronic and long-term inflammation may lead to the development of mucosal atrophy. The failure of gastric immune responses to clear infection is likely to be a result of both bacterial evasion strategies and inappropriate host responses. Host immune responses may be critical in determining the extent of mucosal damage following the onset of chronic infection, and they may also cause perturbations in gastric physiological responses. An understanding of the immunopathology of chronic gastritis will be important in devising new strategies to prevent or modulate gastric *H. pylori* infection.

Acknowledgements

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ROLE IN PEPTIC ULCER DISEASE

6
Acid secretion

JOHN CALAM

INTRODUCTION

Before the importance of *H. pylori* was realized, gastric acid was thought to be the main cause of duodenal ulcers (DU). This was rational because DU patients secrete more acid than controls and suppression of acid both heals and prevents ulcers. At present we know that *H. pylori* causes DU, but it is not clear how it does so. Recent work shows that *H. pylori* causes some of the abnormalities of acid secretion previously described in DU disease. Thus *H. pylori* might produce ulcers by increasing acid secretion. Research has also revealed abnormalities of gastrin and somatostatin physiology which probably underlie the altered acid secretion. This chapter will first summarize the abnormalities of acid secretion and gastrin in DU disease and then describe the extent to which these are explained by *H. pylori* infection.

WORK BEFORE THE DISCOVERY OF *H. PYLORI*

Measurements of acid secretion and the abnormality in DU disease

To understand this area it is necessary to appreciate that acid secretion can be assessed in different ways and under different circumstances. Maximal or peak acid output simply indicates the parietal cell mass. This is quite different from basal and meal-stimulated acid secretion, which reflect the sum of the stimulatory and inhibitory physiological factors prevailing at that time, as well as the number of parietal cells. Intragastric pH and acidity are influenced by all of these factors, plus the buffering capacity of any food present in the stomach. Patients with DU disease tend to secrete more acid than normal individuals[1,2], however acid secretion is measured, although there is always an overlap between rates of secretion in patients and controls.

Maximal acid output and parietal cell mass

Maximal or peak acid output (MAO) is measured during maximal stimulation with pentagastrin or histamine. It reflects the parietal cell mass which is, on

average, 1.5–2 times greater in DU patients than controls[2]. Why do DU patients have more parietal cells than normals? This might be due to the trophic effect of gastrin on parietal cells, an effect which has been clearly demonstrated in animals[1]. However, the rise in gastrin in DU disease[1] is probably insufficient to have such an effect — even gastrinoma patients with much higher gastrin levels have MAOs which overlap with the normal range[3]. Eradication of *H. pylori* diminishes circulating gastrin concentrations in DU disease, but this does not produce a fall in MAO, even 7 and 12 months later[4]. Epidemiological studies based in Finland suggest another explanation. Atrophic gastritis decreases the parietal cell mass in the general population and appears to occur less often in DU patients[5]. Thus the increased parietal cell mass in DU disease might be due to a lack of gastric atrophy rather than the trophic effect of a hormone such as gastrin.

Basal acid output

Patients with DU disease tend to secrete more acid in the fasting or basal state (BAO), both before meals and at night[2]. This is partly because they have more parietal cells, but the BAO/MAO ratio is also increased, so the balance of stimulatory and inhibitory factors appears to be altered in favour of stimulation. This might be due to increased basal gastrin concentrations[1], and increased vagal drive[2], or a decreased number of D cells, which release the inhibitory peptide somatostatin[6]. All of these have been reported in patients with DU disease (see below).

Meal-stimulated acid secretion and the effect of low luminal pH

Postprandial acid secretion is stimulated by cephalic, gastric and intestinal mechanisms which involve neural pathways as well as circulating hormones[2]. However, acid secretion stimulated by amino acids or peptone (protein digested by pepsin) seems to be mostly attributable to gastrin. Meal-stimulated acid secretion in DU patients has generally been found to be increased in parallel with the increase in MAO[2,7]. Interestingly, the duration of the increase in acid secretion which occurs on eating a meal was found to be prolonged in patients with DU disease[2]. This, like the elevation of basal acid secretion, suggests a defect in the mechanism which normally turns off acid secretion. Further evidence of this was provided by the study by Walsh *et al.* who found that a low luminal pH inhibits peptone-stimulated acid secretion less in patients with DU disease than in controls[8].

Measurements of gastrin and the abnormality in DU disease

Studies of circulating hormones in DU disease have focused on the potent acid-stimulating antral peptide hormone gastrin[9]. Elevated basal and post-prandial plasma gastrin concentrations were found in some studies, but not in others[9]. In retrospect this may have depended on whether the controls were infected with *H. pylori* or not (see below). Gastrin release stimulated by infusions of gastrin-releasing peptide was considerably increased in DU

disease. Also, as with acid secretion, the inhibition of peptone-stimulated gastrin release by low intragastric pH was found to be diminished in DU patients[10].

ABNORMALITIES OF GASTRIC PHYSIOLOGY ASSOCIATED WITH *H. PYLORI* INFECTION

When it was discovered that *H. pylori* is an important cause of DU disease it became interesting to ask whether *H. pylori* infection was responsible for the alterations in gastric physiology that had been found in DU patients.

Abnormalities of gastrin in *H. pylori* infection

H. pylori has been found to increase basal and postprandial plasma gastrin concentrations, in both DU and non-ulcer subjects. We first reported this effect in 1989 in a study of DU patients[11], and the effect has since been confirmed by comparing normal individuals with and without *H. pylori*, and by observing a fall in plasma gastrin levels after eradication of *H. pylori* from ulcer and non-ulcer patients[1]. Release of gastrin in response to bombesin and its mammalian equivalent gastrin-releasing peptide are also increased in *H. pylori* infection[12]. Studies of the molecular forms of gastrin have shown that *H. pylori* predominantly increases release of gastrin 17; the form which is most abundant in the gastrin antrum[12]. The gastric antrum is both the site which contains most G cells and the predominant location of *H. pylori* bacteria. Our studies in Los Angeles showed that luminal acid inhibits peptone-stimulated gastrin release less in *H. pylori* infection[13].

Abnormalities of acid secretion in *H. pylori* infection

It should be noted that *H. pylori* has at least three different effects on acid secretion under different circumstances:

1. Initial infection is associated with greatly diminished or absent acid secretion, which lasts for several months. The mechanism is unknown. *H. pylori* produces at least one factor which inhibits parietal cell function[14], but diminished acid secretion is seen in other acute infections, so that some aspects of the inflammatory process might be involved[15].
2. Infection with *H. pylori* probably initiates autoimmune gastritis in some individuals. In this way *H. pylori* can eventually decrease acid secretion[16].
3. In individuals with slight or absent acid gastric atrophy, including DU patients, *H. pylori* increases acid secretion under specific circumstances[13,17,18].

Maximal acid output

Eradication of *H. pylori* has been found not to affect MAO, which reflects the parietal cell mass, even after 7 and 12 months[4].

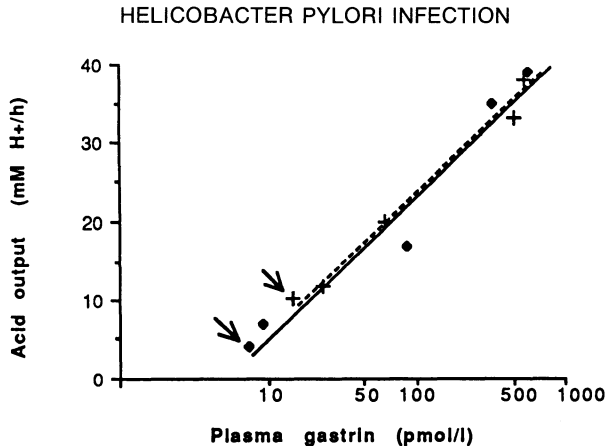

Fig. 1 Acid secretion in response to different doses of gastrin. Plasma gastrin levels were increased by infusing gastrin 17 at different rates: ----, before and ——, after eradication of *H. pylori* from patients with active duodenal ulcer disease. Eradication did not alter the response of parietal cells to gastrin. The arrows indicate the results obtained without exogenous gastrin, before (upper arrow) and after (lower arrow) eradication. Eradication led to significant falls in basal gastrin concentrations ($p < 0.01$) and basal acid secretion ($p < 0.01$)

Basal acid output

We recently showed a fall in mean basal acid secretion from about 12 to about 4 mmol/h on eradication of *H. pylori* from patients with active DU disease[17]. The study included a gastrin–acid dose–response curve. The results suggested that the fall in basal acid secretion was due to a parallel fall in basal plasma gastrin concentrations (Fig. 1).

Meal-stimulated acid secretion; the effect of luminal pH

Meal-stimulated acid secretion has been studied using the technique of intragastric titration: a liquid meal is put into the stomach via a nasogastric tube and the pH is kept constant by adding alkali. The timing of studies appears to be critical in determining the outcome. We initially found no effect of eradicating *H. pylori* on peptone-stimulated acid secretion in a study with DU patients titrated for 1 h each at pH 5.5 and pH 2.5 before and after eradication therapy[19]. It then became apparent that a longer period of titration is required before differences become apparent. In a study performed in Los Angeles we compared non-ulcer volunteers with and without *H. pylori* infection. Each was titrated for 3 h at pH 2.5 and pH 7.0 on separate days. We found that the inhibition of acid secretion produced by the low pH was significantly diminished in individuals infected with *H. pylori* during the 2nd and 3rd hours[13].

Gastrin-releasing peptide-stimulated acid secretion

McColl's group in Glasgow have found that secretion of acid stimulated by intravenous infusion of gastrin-releasing peptide (GRP) is significantly increased in patients with *H. pylori* infection[18]. The effect of GRP on gastric

acid secretion is complex and can involve inhibition of secretion via stimulation of centres in the brain, inhibition of acid secretion by stimulation of somatostatin release from gastric D cells, as well as stimulation of acid secretion via release of gastrin[20]. Therefore, the interesting effect of *H. pylori* on the response to gastrin-releasing peptide could be due to an effect of *H. pylori* on G cells, D cells or nervous pathways.

The role of somatostatin in the inhibition of gastrin and acid secretion

Increases in basal[17], acid-inhibited[13] and immediate postprandial[2] acid secretion in DU disease suggest that there is a defect in a mechanism which normally inhibits acid secretion. Research in experimental animals has shown that gastric D cells play a central role in the inhibition of gastric secretion. D cells are scattered throughout antral and corpus mucosa and release somatostatin locally onto adjacent G cells and parietal cells[20]. Gastric D cells are stimulated by factors which are known to inhibit gastric function. These include inputs from the small intestine: cholecystokinin and the secretin family of peptides (secretin, vasoactive intestinal peptide, glucagon), gastric distension, and a low pH in the gastric lumen.

Gastric D-cell function cannot be studied adequately through measurements of circulating somatostatin, first because this peptide is released in most parts of the gastrointestinal tract and secondly because it acts and is destroyed locally in each region. Therefore studies in animals have employed a variety of techniques including isolated perfused stomachs, somatostatin blocking antisera, cultured D cells and measurements of somatostatin mRNA[20]. For example, perfusion of an isolated mouse stomach with anti-somatostatin antiserum greatly increased acid secretion, indicating that somatostatin exerts tonic inhibition of acid output. A major increase in somatostatin release occurred when the lumen of an isolated perfused pig antrum was acidified. Treatment of rats with omeprazole, which inhibits acid secretion, led to a fall in somatostatin mRNA and a rise in gastrin mRNA. Conversely, in fasted rats somatostatin mRNA rose and gastrin mRNA fell. Fasting produces a low luminal pH by removing the buffering effect of food. Thus fasting and low luminal pH might act in the same way on gastric D cells. These and similar studies lead to the model of D-cell function shown in Fig. 2.

We postulated that increased release of gastrin and acid in *H. pylori* infection is due to decreased D-cell function. There was already some evidence for this from studies showing decreased numbers of D cells and less somatostatin peptide in the gastric mucosa of patients with DU disease. Also Kaneko *et al.*[6] showed less somatostatin peptide in individuals infected with *H. pylori* than in non-infected persons. We wished to study the *function* of gastric D cells. Of the techniques used in animals, the measurement of mRNA seemed most feasible and appropriate. When endocrine cells are stimulated to release peptide their content of peptide mRNA changes in parallel with peptide release, reflecting intracellular mechanisms which coordinate

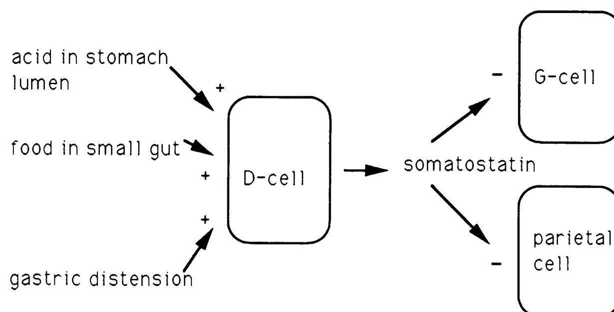

Fig. 2 A model of the role of gastric D cells in the inhibition of G cells and parietal cells in the gastric mucosa. Reference 20 details specific factors which release somatostatin

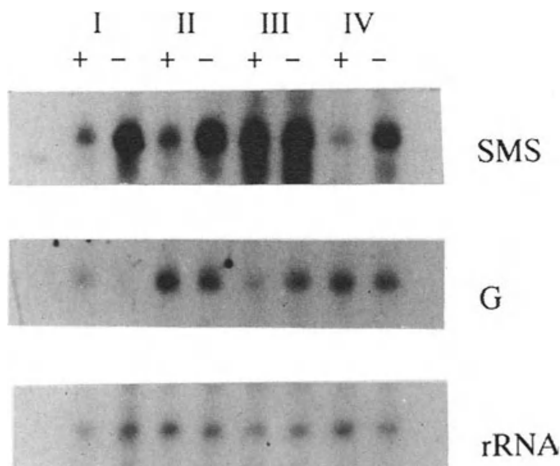

Fig. 3 Northern blot showing somatostatin, gastrin and ribosomal RNAs before (+) and after (−) eradication of *H. pylori* from four patients (I–IV) with active duodenal ulcer disease. A marked rise in somatostatin mRNA occurred in patients I, II and IV

synthesis of new peptide and peptide release. We miniaturized the method for measuring mRNA by Northern blotting to study extracts of five biopsies per patient. Using this technique we found that eradication of *H. pylori* from patients with DU disease led to a significant increase in somatostatin mRNA[21] (Figs 3 and 4). This supports the idea that *H. pylori* inhibits D-cell function.

How does *H. pylori* affect endocrine cells?

We originally speculated that *H. pylori* decreases acid-inhibition of gastric function by neutralizing the mucosal microenvironment, through alkali production by its abundant enzyme urease[11]. However, subsequent studies

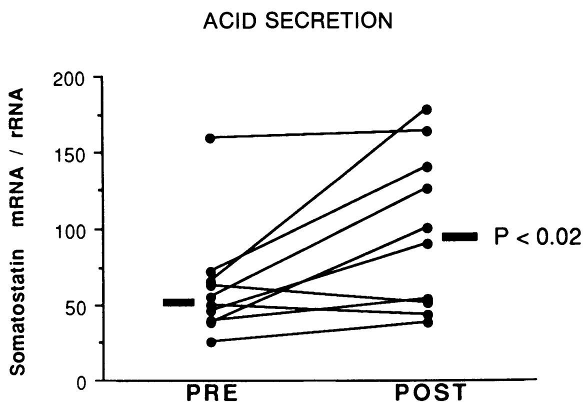

Fig. 4 Scattergram showing the rise in the somatostatin mRNA/rRNA ratio on eradication of *H. pylori* from patients with active DU disease ($p < 0.01$)

by ourselves and by Dr McColl's group do not support this idea[1]. The antral mucus layer was more alkaline, but only marginally so[23], and neither instillation of excess urea into the stomach nor administration of a urease inhibitor[22] altered plasma gastrin levels. It now seems more likely that the inflammatory process in the antral mucosa leads to the altered function. In a series of studies performed in Los Angeles we examined the effect of human peripheral blood mononuclear cells and live *H. pylori* bacteria on gastrin release from a mixture of canine G and D cells in primary culture. *H. pylori* significantly increased gastrin release, but only in studies where direct contact between the bacteria and the cells was possible. The mononuclear cells had a stronger effect which persisted when direct contact was prevented, indicating that diffusible factors were responsible. Two pure cytokines, tumour necrosis factor-α and interferon-γ, also released gastrin from the preparation[24]. The studies are to be repeated with measurement of somatostatin. Several other workers, notably Teichmann, have reported that these and various other cytokines can release gastrin *in vitro*[1].

SUMMARY AND CONCLUSIONS

Current evidence supports the following ulcerogenic mechanism in *H. pylori* infection.

1. *H. pylori* bacteria attract and activate leucocytes which release cytokines.
2. Cytokines inhibit somatostatin cells (D cells) and stimulate gastrin cells (G cells).
3. Decreased expression of somatostatin and increased gastrin release lead to excessive acid secretion, particularly at times when acid secretion would normally be inhibited.
4. Increased acid secretion promotes ulcerogenesis, particularly in individuals with a higher parietal cell mass, perhaps due to a lack of atrophic gastritis.

However, it should be noted that many of these effects of *H. pylori* are seen in individuals with *H. pylori* but without ulcers and in DU patients between episodes of ulceration. Therefore, other factors such as bacterial toxins or the susceptibility of the host remain highly interesting topics for further research[25].

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7
Mucosal defence

PATRICK GOGGIN AND TIM NORTHFIELD

INTRODUCTION

The recognition that impairment of mucosal defence might play a part in the pathogenesis of peptic ulceration predates the identification of *H. pylori* by more than 70 years. In 1910 Schwarz hypothesized that peptic ulcer is a product of self digestion, which results from a 'disproportion in the normal balance between the autopeptic power of gastric juice and the protective forces of the gastric mucous membrane'. Since this time various abnormalities of mucosal defence have been identified in peptic ulceration, but their importance in pathogenesis has remained unclear (Fig. 1).

The identification of *H. pylori* as a necessary cofactor in the pathogenesis of most cases of peptic ulcer has led to a re-examination of the subject, and has provided a pathogen which may explain observed abnormalities. In addition, other abnormalities of mucosal defence have been identified in association with *H. pylori* infection, which had not previously come to light.

The components of the mucosal defence system comprise an intricate combination of anatomical entities such as the mucus layer and the apical cell membrane, and physiological processes such as mucosal blood flow, epithelial cell restitution, bicarbonate secretion and prostaglandin synthesis. Some measurements of mucosal defence such as juxtamucosal pH may be the product of several of these individual factors.

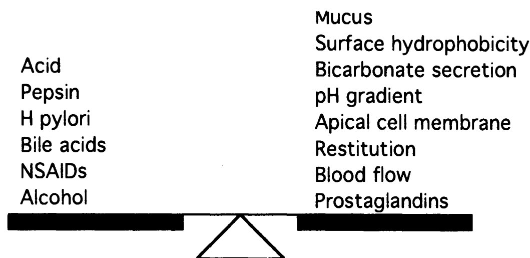

Fig. 1 Balance of aggressive and defensive factors at the mucosal surface

The aims of this chapter are to review the abnormalities in mucosal defence that have been identified in peptic ulcer disease, to determine which of these are associated with *H. pylori* infection and to examine the possible mechanisms. That many of the pathogenic effects of *H. pylori* are essentially the result of reduced resistance to attack by pepsin and acid is amply demonstrated by the observation that many ulcers occur in patients who are not hypersecretors of acid, and that most ulcers will heal and not recur simply with suppression of acid.

THE MUCUS LAYER

The mucus layer has long been thought to play an important protective role in the stomach, both as a lubricant and as part of the gastric mucosal barrier against acid and pepsin, though its precise role in mucosal defence remains controversial, and it is not even clear whether it forms a continuous layer. Mucus comprises a complex mixture of glycoproteins, lipids and glycolipids, each contributing to its structure and physical properties. *H. pylori* is found both free within the mucus and adherent to the mucus-producing cells, and is therefore ideally suited to affect this layer.

A pH gradient across the mucus layer has been demonstrated using microelectrodes in animal studies and in studies on human gastric resection specimens, with the juxtamucosal pH remaining close to neutral down to a luminal pH of around 2. In the antrum the gradient may be overwhelmed at a much higher pH. Quigley and Turnberg[1] measured juxtamucosal pH using a glass electrode passed down the biopsy channel of an endoscope, and found that in the duodenum a pH gradient across the mucus layer was maintained down to a luminal pH of 1.5 or less. Patients with duodenal ulcer were found to have a lower than normal luminal and juxtamucosal pH. When acid secretion was controlled for by infusing a buffer at pH 2, mean juxtamucosal pH remained lower in the duodenal ulcer patients compared to controls in the body (3.4 vs 5.5), antrum (4.2 vs 5.4) and duodenal cap (5.7 vs. 6.7). Quigley and Turnberg did not determine the presence of *H. pylori* in these subjects, so it is not possible to say from this study whether the differences are related to *H. pylori* status. Tally *et al.*[2], using the same methodology, measured the gastric mucus pH gradient in controls and non-ulcer dyspepsia subjects and found no difference between *H. pylori* positive and negative subjects, suggesting that the abnormalities demonstrated by Quigley and Turnberg were not related to *H. pylori* infection but to some other abnormality in duodenal ulcer subjects. By contrast, Kelly *et al.*[3], again using similar methodology, found that in the antrum the mean juxtamucosal pH in 28 patients negative for *H. pylori* was 6.40 compared to 6.88 in 19 patients who were *H. pylori* positive ($p < 0.0001$), i.e. an increase in juxtamucosal pH in patients who are infected with *H. pylori*. In six patients in whom *H. pylori* was subsequently eradicated, mean pH fell from 6.81 to 6.08 ($p < 0.001$). This observation is put forward as indicating that ammonia production by the organism is capable of altering the gastric mucus microclimate to impair the normal negative feedback controlling gastrin

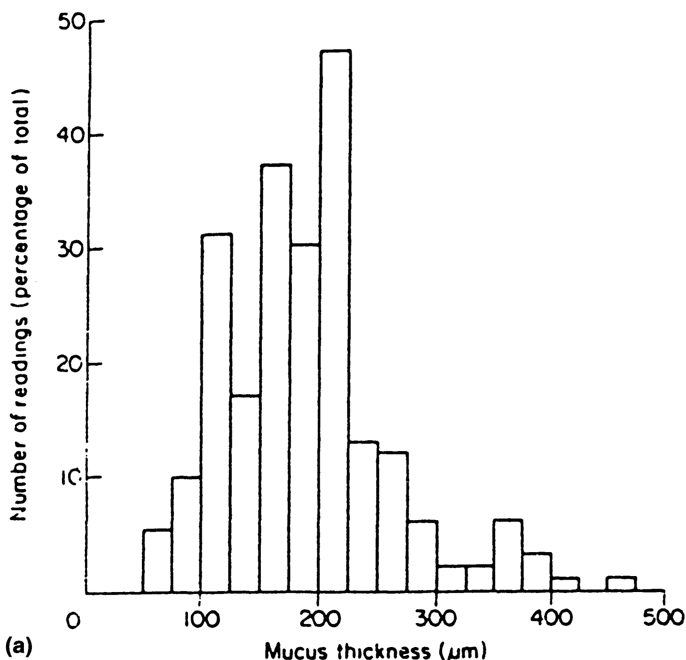

Fig. 2 Adherent mucus gel thickness on human gastric mucosa in (**a**) controls, and (**b**) patients with duodenal ulcer. (Reproduced from Allen *et al.*[4])

release. Thus the evidence appears contradictory. This may reflect the large size of the pH electrode (1–1.3 mm) used, the diameter of which is more than 3 times the depth of the unstirred layer. To get to the juxtamucosal space the electrode must push the mucus out of the way, but once it is in place the electrode itself is a barrier to diffusion of hydrogen ions from the lumen so that the unopposed secretion of bicarbonate or ammonia production by *H. pylori* will raise the pH above that which is present in the static situation. Thus a pH electrode of this type is more likely to be sensitive to changes in apical cell permeability and bicarbonate secretion than to the quality of the mucus layer. This problem does not arise with the microelectrodes used in animal and human *in-vitro* studies. It remains possible that *H. pylori* significantly impairs the ability of the mucus layer to maintain a pH gradient, particularly in the duodenum.

With respect to the depth of the mucus layer, Allen *et al.*[4] used dark-field and phase-contrast illumination to measure the thickness of the mucus layer in antral mucosa of gastrectomy specimens from controls and patients with duodenal ulcer and gastric ulcer. In the controls, median thickness was 180 μm, and no areas had a depth less than 50 μm, while in duodenal ulcer patients the median thickness was 110 μm with a minimum thickness as low as 10 μm (Fig. 2). In gastric ulcer patients the mucus layer had a very different appearance, containing large amounts of cellular debris, making it difficult to detect the demarcation between mucus and mucosa, but the median

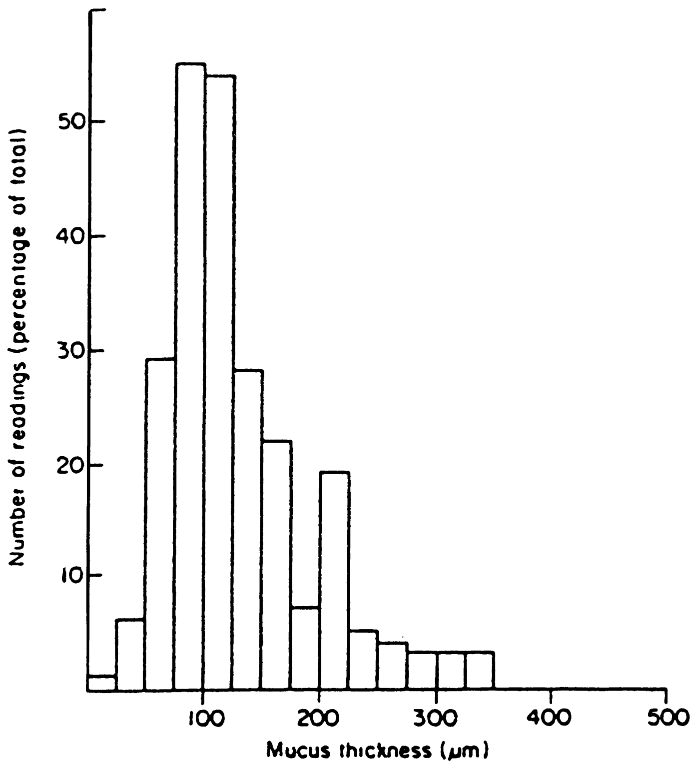

Fig. 2 (b)

thickness appeared similar to controls. That this abnormality is probably related to *H. pylori* infection is suggested by a more recent study by Sarosiek *et al.*[5], who used a similar method on fresh biopsy specimens obtained at endoscopy. In patients with confirmed *H. pylori* infection compared to *H. pylori* negative subjects, the mean thickness of the mucus layer was reduced in the duodenum (93 vs 162 μm), antrum (85 vs 175 μm) and body (105 vs 161 μm) (Fig. 3). Although the effect of eradication on mucus gel thickness has not been studied, it appears likely that the reduction in mucus gel thickness is an effect of *H. pylori.*

The thickness of the mucus layer is itself a complex function of the quantity and quality of the mucus secreted in terms of viscosity, gel formation and ability to resist the digestive powers of pepsin and acid. Younan *et al.*[6] found that antral mucus from gastric resection specimens obtained from patients with duodenal and gastric ulcer contained a higher proportion of low molecular weight glycoproteins (50% and 65% respectively) compared to controls without ulcer (33%), suggesting that the quality of the mucus is impaired in peptic ulcer disease. The proportion of polymeric mucus has been shown to be closely related to the quality of the mucus gel[7] in terms of its viscoelastic properties. It is unclear from these studies whether the

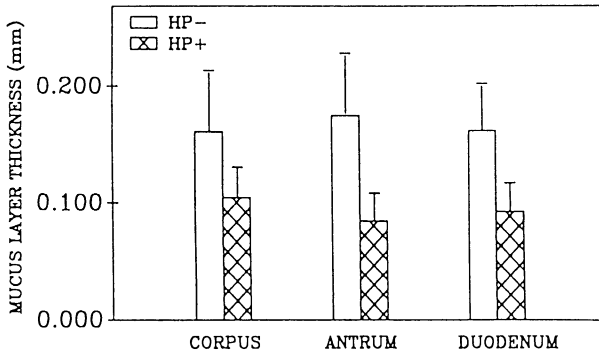

Fig. 3 Adherent mucus gel thickness measured on human gastric biopsy specimens from non-ulcer dyspeptic patients with or without *H. pylori* infection. (Reproduced from Sarosiek *et al.*[5])

changes in peptic ulcer disease are related to *H. pylori* infection and whether they are due to secretion of incompetent mucus or to its degradation after secretion. The greater changes in gastric ulcer, in which *H. pylori* infection is less common than in duodenal ulcer, would suggest that *H. pylori* infection alone cannot account entirely for the abnormality, though there is growing evidence that *H. pylori* has a major effect on mucus production and secretion.

Light microscopic and ultrastructural[8] studies indicate that *H. pylori* infection is associated with depletion of mucus granules in the surface mucus cells. However Baczako *et al.*[9], studying mucosal biopsy specimens in tissue culture, found that incorporation rates of ^{3}H-glucosamine into tissue and the secretion of ^{3}H-labelled mucus were increased in patients with chronic active gastritis, suggesting that mucus depletion may reflect an increased rate of secretion. Liau *et al.*[10], examining the effect of *H. pylori* lipopolysaccharide on the synthesis and secretion of mucin in gastric mucosal segments in tissue culture, found that while there was no discernible effect on apomucin synthesis, glycosylation and sulphation were inhibited by up to 65%. Secretion of mucin was initially stimulated, but was subsequently inhibited. Reduced sulphation may reduce the resistance of mucus to proteolytic degradation by pepsin. Thus it appears that the quality of glycoprotein secreted is impaired by *H. pylori* infection and that this may be related to the presence of lipopolysaccharide. The long-term effect of *H. pylori* infection *in vivo* on the level of mucus synthesis and secretion by gastric mucosa may be different from the short-term effect *in vitro*, as a result of indirect factors such as the development of chronic active gastritis.

There is also evidence that *H. pylori* may degrade glycoprotein after secretion by the production of a protease enzyme. Slomiany *et al.*[11] reported that a cell free filtrate of *H. pylori* degraded mucus glycoprotein into similar-sized fragments to those produced by pepsin, and reduced mucus viscosity and its ability to retard the diffusion of hydrogen ions. Other groups have failed to identify this enzyme. Baxter *et al.*[12] reported that the proteolytic activity must be at least 1000 times lower than that detected by Slomiany. Sidebotham *et al.*[13] were similarly unable to detect significant proteolytic

activity, and suggested that the experimental findings were due to the direct effects of ammonia following hydrolysis of urea by *H. pylori* urease, though experimental findings were due to the direct effects of ammonia following hydrolysis of urea by *H. pylori* urease, though experimentally added 2 mol/l urea was necessary to produce this effect, while the urea concentration in the stomach is a few millimoles. Thus the existence of *H. pylori* protease remains controversial at present.

Neutral lipids and phospholipids also appear to be an important functional component of the mucus layer. *In vitro* removal of these components leads to a reduction in viscosity and in the ability of mucus to inhibit the diffusion of hydrogen ions[14]. There is evidence that *H. pylori* produces both neutral lipase and phospholipase enzymes. Slomiany *et al.*[15] found that a cell free filtrate of *H. pylori* has phospholipase A2 and lipase activity against phosphatidylcholine and triglycerides, the main products of lipolysis being lysophosphatidylcholine and monoglycerides respectively. This activity is inhibited by bismuth and sucralfate[16]. Langton and Cesaro[17] found gastric juice phospholipase A2 activity to be higher in infected subjects, though it is unclear whether this is of bacterial or mucosal origin since the mucosa itself has phospholipase A2 activity[18]. In addition to the detrimental effects of the loss of lipids, lysophosphatidylcholine has been shown to reduce mucus viscosity[19] and is a barrier breaker which will increase the fluidity of the apical cell membrane and increase back diffusion of hydrogen ions.

SURFACE HYDROPHOBICITY

The water-repellent or hydrophobic nature of the gastric mucosa has been proposed as a mechanism of mucosal defence, on the basis of animal studies[20] showing that the gastric mucosa forms a high contact angle with saline drops applied to the surface. In addition, damaging agents such as bile acids and NSAIDs have been demonstrated to reduce surface hydrophobicity, while prostaglandins reverse these changes[20,21]. We have validated a method for measurement of surface hydrophobicity in man on endoscopic biopsy specimens using a contact angle technique[22]. Mean contact angle of the gastric body and antrum was 70°, indicating a relatively low energy or hydrophobic surface.

In duodenal ulcer and gastric ulcer disease[23] antral contact angle is reduced (57° and 59° respectively) regardless of the activity of the ulcer, and in duodenal ulcer disease there is a similar reduction in the contact angle of the duodenal bulb (52° vs 62° for controls). Subjects with *H. pylori* positive gastritis[23] show a similar reduction in gastric mucosal hydrophobicity to that seen in peptic ulcer disease, while *H. pylori* negative gastritis is not different from controls (Fig. 4), suggesting that *H. pylori* may be responsible for the abnormality, and that it may be a direct effect of *H. pylori* infection rather than related to the presence of gastritis.

To clarify this further, we determined the effect of clearance and eradication of *H. pylori* in patients with and without duodenal ulcer[24]. Following treatment with ranitidine contact angle was unchanged (55° vs 56°) but it

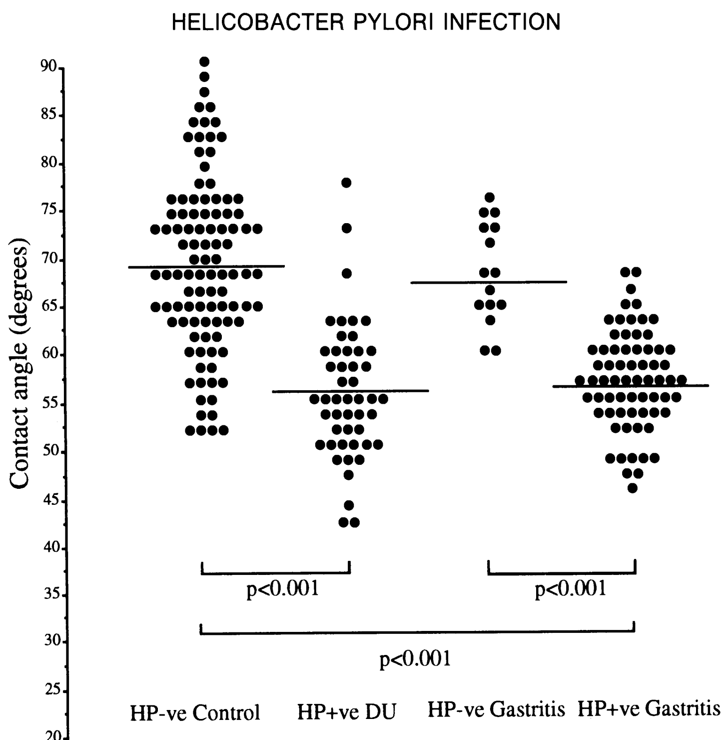

Fig. 4 Contact angles of antral mucosa in active DU and GU subjects and in non-ulcer controls. (Reproduced from Spychal *et al.*[23])

increased with bismuth (57° to 52°, $p < 0.05$) and bismuth plus antibiotics (56° to 67°, $p < 0.0001$). One month after treatment ended, contact angles in patients with persistent infection were not different from those before treatment (56° vs 56°), but increased to a value similar to *H. pylori*-negative controls in patients in whom *H. pylori* was eradicated (56° to 69°, $p < 0.0001$) (Fig. 5). After eradication of the organism, contact angles in the ulcer group returned to a value similar to that of *H. pylori*-negative controls who had no history of ulceration. Thus there does not seem to be an underlying defect in surface hydrophobicity in peptic ulcer patients, other than that caused by the presence of *H. pylori* infection.

The mechanism by which *H. pylori* reduces gastric mucosal hydrophobicity is more difficult to ascertain since contact angle measurement does not provide specific information about the molecular configuration at a tissue surface[25,26]. The mechanism could involve the organism itself within the mucus layer either exerting a direct topical effect that makes the surface appear more hydrophilic, or causing an indirect toxic effect due to release of proteases[11] or phospholipases[27] that could alter surface hydrophobicity.

Structural[28] and physical[29] studies have shown gastric mucosal hydrophobicity to be a property of the surface mucus layer and to be related to the phospholipid content of this layer. In particular, increased phosphatidylcholine is associated with an increase, and lysophosphatidylcholine with a

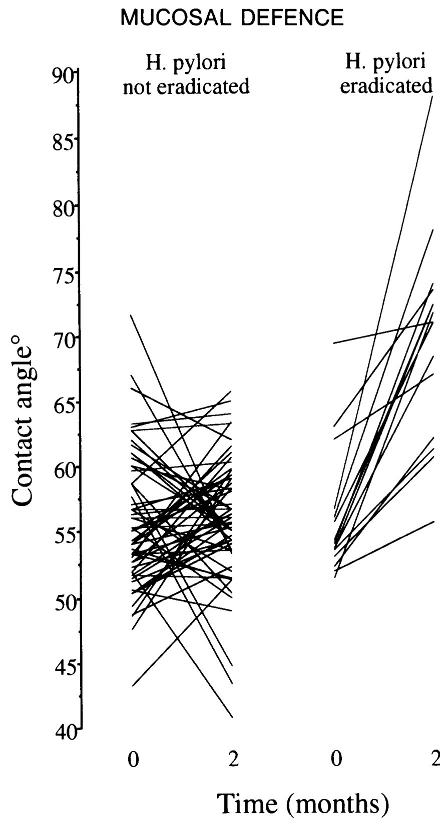

Fig. 5 Antral contact angle before and 1 month after cessation of treatment in patients who eradicated or failed to eradicate *H. pylori*. (Reproduced from Goggin *et al.*[24])

reduction in surface hydrophobicity, suggesting a possible mechanism for the action of *H. pylori* via production of its phospholipase A2 enzyme. This mechanism is supported by the observation of increased phospholipase activity in gastric aspirates of *H. pylori*-infected patients mentioned above. The abnormality is unlikely to be due to changes in mucus layer thickness in *H. pylori* infection, since the measurement has been shown to be independent of the thickness of a mucus layer[29].

THE APICAL CELL MEMBRANE AND TIGHT JUNCTIONS

Beneath the mucus layer the apical cell membrane and tight junctions form an important barrier to the diffusion of acid. Animal studies have shown the apical cell membrane to have a high content of cholesterol and unsaturated fatty acids suggesting a relatively stiff membrane which would be expected to have a low permeability to hydrogen ions. Lysophosphatidylcholine produced by *H. pylori* phospholipase A2 might be expected to increase the permeability of this membrane, allowing the diffusion of bicarbonate and nutrients to the advantage of the organism. The precise lipid composition of

the apical cell membrane has not been studied in humans. Nardone *et al.*[30] measured the phospholipid composition of whole human gastric biopsy specimens, and found that patients with duodenal ulceration (the majority of whom would be expected to be infected with *H. pylori*) had lower levels of phosphatidylinositol and higher levels of phosphatidylcholine than controls. The likely effect on membrane fluidity and permeability is unclear. Ultrastructural studies[8] demonstrate *H. pylori* infection to be associated with loss of the normal microvillus structure of the apical cell membrane and disruption of the intercellular junction complexes, though no physiological studies have been performed to show whether this is associated with a change in permeability.

Transmucosal potential difference is in part a reflection of the ion permeability of the apical cell membrane, and has been used as a measure of mucosal defence. Its use as such is supported by the observation that barrier breakers such as bile and aspirin acutely reduce transmucosal potential difference. In the ischaemic *ex-vivo* rat stomach the combination of *H. pylori* and urea has been shown to reduce transmucosal potential difference[31], but in a group of non-ulcer dyspeptic patients Pfeiffer *et al.*[32] found no difference in gastric mucosal potential difference between those with and without *H. pylori* infection.

PROSTAGLANDINS

Prostaglandins have a wide range of effects on the components of the gastric mucosal barrier, protecting the mucosa against noxious substances, though they are not truly *cytoprotective*. These actions include stimulation of mucus and alkaline secretion, enhancement of gastric mucosal blood flow, dissolution of gastric mucosal folds, and stimulation of surface-active phospholipids. The effect of *H. pylori* on prostaglandin and leukotriene production is somewhat uncertain. Fukuda *et al.*[33] reported LTB4 increased in *H. pylori*-associated gastritis, but the concentration correlated with the degree of neutrophil infiltration suggesting that it may be an epiphenomenon rather than a pathogenic effect. Taha *et al.*[34], studying patients with benign gastric ulcer and non-ulcer dyspeptic controls, found increased prostaglandin synthesis in severe gastritis irrespective of the presence of *H. pylori* infection, suggesting that the increased prostaglandin production is a feature of the inflammation rather than a direct effect of *H. pylori*. By contrast Cryer *et al.*[35], measuring prostaglandin concentration rather than production, found that in healthy volunteers there was no relationship between prostaglandin levels and *H. pylori* infection or the degree of inflammation. Taha *et al.*[36] studied the effect of *H. pylori* proteins on cAMP and PGE_2 production in incubates of human gastric fundic mucosa and found that although *H. pylori* protein alone had no effect on PGE_2 production, cAMP production was reduced 3-fold and the stimulatory effect of histamine on both PGE_2 and cAMP production was prevented. Given the role of cAMP in various physiological responses, this suggests that *H. pylori* might alter those

functional aspects of the human gastric mucosa which rely on cAMP as a second messenger, and blunt the prostaglandin response to injury.

MUCOSAL BLOOD FLOW

Adequate mucosal blood flow is an important factor in mucosal defence, and numerous studies have demonstrated that reduced blood flow can lead to ulceration or enhance the effect of other ulcerogens. In humans, laser Doppler flowmetry has demonstrated that NSAIDs reduce mucosal blood flow to 75% of control, but no studies have examined specifically the effect of *H. pylori*. Lunde *et al.*[37] found mucosal blood flow reduced in the region of the ulcer in patients with lesser curve gastric ulcers (the majority of whom would be expected to be infected with *H. pylori*) but not in any other part of the stomach, when compared to younger control patients. This suggests that *H. pylori* infection may have little effect on mucosal blood flow *per se*, but further studies are needed for this to be confirmed.

EPITHELIAL CELL RESTITUTION

In vivo re-epithelialization of the gastric epithelium following damage by a noxious agent can occur within a few hours. This process takes less time than is required for cell division, and is dependent upon the initial formation of a fibrin-mucoid cap with increased secretion of bicarbonate followed by migration of cells from the gastric pits and from adjacent areas. Bui *et al.*[38] found that, following production of an experimental gastric ulcer in rats, twice-daily ingestion of a suspension of *H. pylori* greatly reduced ulcer healing rate, and also reduced the quality of healing. Thus *H. pylori* appears to interfere with the normal healing process, perhaps by a cytotoxic effect on the migrating gastric mucosal cells.

CONCLUSIONS

H. pylori infection has been shown to be associated with a number of defects in mucosal defence. Three of these defects are associated with peptic ulceration. One defect is a reduction in mucosal hydrophobicity, indicating a reduced ability to repel aqueous solutions including acid. A second defect is a thinner mucus gel layer, which appears to be an independent effect from hydrophobicity. The mucus gel layer is also abnormal in structure, with an increased proportion of low molecular weight glycoproteins. This may render it a less effective barrier to the diffusion of pepsin. The third defect is a reduced pH gradient across the mucus layer, which could result from the reduced hydrophobicity or from the thinner mucus gel layer.

The reduction in mucosal hydrophobicity is related to the presence of *H. pylori* infection, and is reversed by successful eradication therapy. The presence of *H. pylori* infection has also been reported to be associated with a thinner and weaker mucus gel structure. In some but not all studies, an

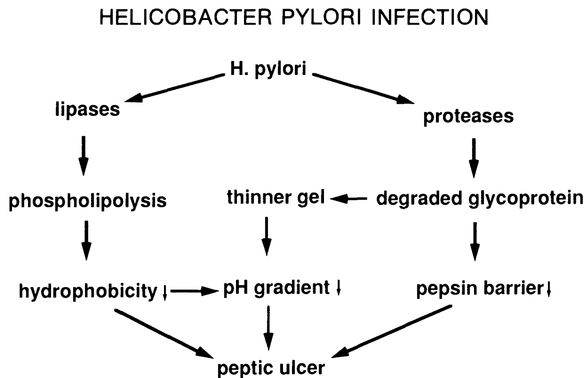

Fig. 6 Overall concept linking *H. pylori* infection with peptic ulcer formation, via impaired mucosal defence

association with a reduced mucosal pH gradient has also been reported.

In vitro, the *H. pylori* organism has been reported to produce lipases and proteases. The lipases include phospholipase A2, which converts lecithin, a hydrophobic molecule, into lysolecithin, a detergent molecule. The mucus gel layer contains phospholipids, especially lecithin, which could contribute to the hydrophobic barrier, but which could be broken down to lysolethicin, a barrier breaker. Phospholipase A2 activity and lysolecithin levels have been reported as raised in gastric aspirate of *H. pylori*-infected subjects. Proteases are capable of degrading glycoprotein, and could thus cause a thinning of the mucus gel layer and a reduction in the barrier to pepsin.

Figure 6 gives an overall concept that links *H. pylori* infection with the pathogenesis of peptic ulceration via these reported defects in mucosal defence. At present this concept is best regarded as a working hypothesis, and further studies are necessary to confirm the interrelationships. What does seem certain is that the measurement of mucosal hydrophobicity provides a quantitative method of measuring mucosal defence in humans; that this measurement is impaired in patients with peptic ulcer; and that this impairment is due to the presence of *H. pylori* infection.

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ROLE IN PEPTIC ULCER DISEASE

8 Overview

GUIDO TYTGAT and MICHAEL DIXON

INTRODUCTION

The clinical expression of chronic *Helicobacter pylori* (Hp) infection in humans is highly variable. Most infected individuals are asymptomatic. A minority suffers from dyspeptic symptoms and/or duodenal and gastric ulcer disease. Finally, gastric adenocarcinoma and primary gastric lymphoma are increasingly considered as potential end-stage consequences of chronic Hp-associated inflammation.

It is highly controversial whether at present Hp-infected patients without, or even with, dyspeptic symptoms deserve anti-Hp therapy. It is unclear when dyspeptic symptoms are genuinely related to the Hp-associated inflammation. As the anti-Hp therapy is still evolving, most investigators argue for restraint, and advise treating dyspeptic patients within the framework of a scientific trial. How to conduct such a dyspepsia study, which symptoms to monitor and for how long, and which drugs to use is very unclear at the present time. Guidelines for proper design and conduct of dyspepsia trials will hopefully soon become available.

There is increasing consensus in the literature that the best current indications for anti-Hp therapy concern genuine Hp-associated duodenal ulcer disease (DU) and genuine non-drug-associated gastric ulcer disease (GU). Especially for DU, much less so for GU, evidence has been provided that Hp-eradication dramatically reduces their natural tendency to relapse. This overview will therefore be limited to the current indications and results of anti-Hp therapy in DU and GU.

DUODENAL ULCER DISEASE

Prevalence of *H. pylori* infection in duodenal ulcer disease

The prevalence of *H. pylori* infection has consistently been found to be between 95% and 100%[1]. Of over 250 DU patients studied over the past few years in Amsterdam, the Hp infection rate was 100% if we eliminate one

patient who had taken antibiotics in the recent past. A DU occurring in a patient not infected with Hp is not genuine peptic ulcer disease, and some other cause (NSAIDs, Crohn's disease, gastrinoma, etc.) should be sought[2]. A clear-cut direct causal relationship has been accepted by the scientific community between Hp infection and gastritis, but such a direct relationship does not exist as yet between Hp and DU. Only a minority of individuals harbouring Hp will develop a DU with a lifetime prevalence of around 10%. Hp therefore plays the role of a risk factor, albeit the most dominant one, because sustained eradication in essence cures the disease.

Histopathological aspects of duodenal ulcer disease

The gastritis associated with DU predominantly affects the antrum. The antral mucosa shows superficial or full-thickness infiltration by lymphocytes and plasma cells with occasional lymphoid follicles. There is almost invariably a neutrophil polymorph component either within the lamina propria or infiltrating the foveolar and surface epithelium. Also, atrophy with intestinal metaplasia of the antrum can be found in many DU patients[3–6]. In some there is also inflammation of the corpus, albeit to a lesser degree compared to the antrum. A diffuse antral gastritis with sparing of corpus mucosa means that the parietal cell mass is unaffected and permits normal, or even increased, acid production. Why there is relative sparing of the corpus mucosa is largely unknown. High corpus acidity perhaps inhibits bacterial growth and restricts the infection mainly to the antrum.

The strong association between gastritis and duodenal ulceration does not by itself prove a causal role for the former. However, epidemiological and follow-up studies point to gastritis preceding ulceration. Sipponen *et al.*[7] reported on a 10-year follow-up of individuals with and without gastritis. Only one out of 133 (0.8%) individuals with a normal gastric histology at entry developed a peptic ulcer (DU/GU). In contrast, 29 out of 233 (12.4%) individuals with antral gastritis developed ulcers. On the basis of this and other studies it appears that, although chronic gastritis is common in the community, only 10–15% will ever develop a symptomatic peptic ulcer.

There is a close association between chronic duodenitis and the subsequent development of an ulcer crater. A consistent histological finding in duodenitis is surface gastric metaplasia, the appearance of patches of gastric-type mucous cells interspersed between absorptive and goblet cells of the duodenal epithelium[8–10]. Gastric metaplasia is not restricted to duodenitis, but can be found in healthy individuals, but usually to a more limited degree. Evidence is accumulating from experimental and human studies indicating that duodenal bulb acidity appears to be the most important determinant for the development of gastric metaplasia[9]. That gastric metaplasia is an acquired change seems indisputable, but whether the metaplasia represents divergent differentiation or repopulation by cells migrating from the ducts of Bruner's glands remains unknown.

The pathogenic link between Hp infection and chronic gastritis, with or without glandular atrophy, is irrefutable. A similar role for Hp in the

aetiology of chronic duodenitis has been more difficult to establish. Infection by Hp appears restricted to gastric epithelial cells. The presence of gastric metaplasia would allow such colonization to occur in the duodenum, and close interrelations between gastric metaplasia, duodenitis and Hp-associated gastritis have indeed been established. Johnston *et al.*[11] found that Hp could be seen in the duodenum of virtually all patients with active duodenitis or ulcer, but not in those with normal findings or inactive inflammation. However, not all authors have been so successful.

In summary, Hp tends to colonize both antrum and corpus, but active inflammation is often limited to, or most pronounced in, the antrum[12,13]. The acid-secretory portion of the stomach is relatively resistant to infection, especially in DU patients. This may simply reflect a hostile environment because of hyperacidity. Thus increased acid secretory capacity (genetically determined?) could explain both tissue responses to Hp infection, being antral restriction of gastritis, and presence of gastric metaplasia in the duodenum, the two principal features distinguishing DU from GU. As a result of chronic active inflammation in the bulb, mucosal resistance is impaired and acid-peptic attack on the weakened mucosa leads to erosive duodenitis and ultimately to frank ulceration.

Bacterial characteristics in duodenal ulcer disease

Duodenal ulcer disease is obviously a multifactorial event. In addition to the microbial gastroduodenitis there are many other disease modifiers such as acid-pepsin secretory capacity, smoking, other genetic and/or environmental factors. etc. The interplay of these various and variable risk factors might explain why some Hp-infected individuals develop a DU, while others with apparently comparable degrees of inflammation and acid-peptic secretory capacity do not, or why DU relapses occur periodically against the background of a constant acid secretory capacity and a stable bacterial infection. Alternatively, bacterial strains may differ with respect to their ulcerogenic potential. A vacuolizing cytotoxin, producing vacuolization of cultured epithelial cells, is more commonly present in Hp isolates from DU patients[14–18]. Furthermore, an association between a microbial protein component and the severity of gastritis seems demonstrable in DU patients[19]. This 120 kDa protein appears to be associated with evidence of more severe gastritis and with peptic ulcer disease. Therefore, this 120 kDa protein may perhaps be considered as a marker for a particular bacterial factor or for a particular Hp–host interaction leading to more severe gastritis and ultimately peptic ulcer disease.

Furthermore, there is evidence of a gene (cag A) which is present in Hp strains associated with peptic ulcer disease[20]. Moreover also genetic studies using DNA/DNA hybridization in solution have shown that Hp strains, obtained from volunteers with asymptomatic gastritis, are in a different hybridization group from strains isolated from DU patients[21]. Again this would be compatible with strain differences between ulcer and non-ulcer isolates. Recent studies add support to this concept. The ability of an Hp

Table 1 Effects of adjuvant antimicrobial therapy on duodenal ulcer healing rate

	n	*Therapy*	*Duration (weeks)*	*Percentage healing*
Bayerdörffer *et al.*, 1987[25]	50	RAN vs RAN + ofloxacin	4	68 vs 92
Marshall *et al.*, 1988[26]	100	CBS vs CBS + tinidazole	10	68 vs 74
		CIM vs CIM + tinidazole	10	59 vs 76
Graham *et al.*, 1992[27]	105	RAN vs RAN + BSS + tetracycline + metronidazole	16	84 vs 98
Mannes *et al.*, 1993[28]	178	RAN vs BSS vs BSS + amoxicillin	6	69 vs 77 vs 84
Hentschel *et al.*, 1993[29]	104	RAN vs RAN + amoxicillin + metronidazole	6	75 vs 92

RAN = ranitidine; CIM = cimetidine; CBS = colloidal bismuth subcitrate; BSS = bismuth subsalicylate

strain to produce ulcers appears to correlate with its alcohol dehydrogenase activity[22], with a lower capacity to inhibit acid production from parietal cells *in vitro*[23] and with a markedly increased gastrin-mediated acid secretion[24].

Effect of *H. pylori* suppression/eradication on duodenal ulcer healing

Acceleration of ulcer healing by antibacterial therapy would support the role of Hp-associated inflammation in the DU diathesis. The limited number of studies is summarized in Table 1. The amalgamated data on ulcer healing kinetics suggest that adjuvant antimicrobial therapy may indeed accelerate DU healing. Such data suggest that Hp not only plays a role in the pathogenesis of DU relapse but is also an important factor in the healing process. However, more data are necessary to appreciate the clinical relevance of antibiotic-induced, accelerated DU healing.

Effect of *H. pylori* eradication on duodenal ulcer relapse

Elimination of Hp has been clearly shown to alter the natural history of duodenal ulcer disease. Several studies summarized in Table 2 have now shown that ulcers recur in only a very small percentage when Hp is eradicated, compared with 80% or more where the organism remains present. Some of the higher relapse figures after Hp eradication may be explained by either an insufficient number of biopsies taken, sampling mucosa of the antrum only and monitoring relapse only, based upon symptoms instead of endoscopy, as happened in Marshall's study. Where ulcers do recur, it may be due either to recrudescence of infection where 'eradication' was incomplete, or to genuine reinfection. In one study it was shown that recurrence of the organism occurs before the ulcer relapses[55].

It has been argued that the lower relapse rate is not the result of Hp eradication, but due to the use of bismuth as one of the treatment modalities. This is unlikely to be the case, because in the Amsterdam study 50 individuals

Table 2 Duodenal ulcer (DU) relapse rate in patients with persistent *H. pylori* (Hp+) infection and after eradication of *H. pylori* (Hp)

Study	n	Follow-up (months)	DU-relapse Hp+	DU-relapse Hp−
Coghlan *et al.*, 1987[30]	39	12	22/ 29 (76%)	1/10 (10%)
Lambert *et al.*, 1987[31]	45	6	25/ 33 (76%)	0/12 (0%)
Marshall *et al.*, 1988[32]	70	12	38/ 47 (81%)	5/23 (22%) (symptomatic)
Smith *et al.*, 1988[33]	36	18	20/ 29 (69%)	0/ 7 (0%)
Borody *et al.*, 1988[34]	21	12–25	3/ 3 (100%)	0/18 (0%)
Borody *et al.*, 1989[35]	58	9–37	3/ 4 (75%)	0/54 (0%)
Rauws *et al.*, 1990[36]	38	12	17/ 21 (81%)	0/17 (0%)
Blum *et al.*, 1990[37]	192	6	73/179 (41%)	1/13 (8%)
George *et al.*, 1990[38]	62	12–48	–	0/62 (0%)
Grigorjev *et al.*, 1990[39]	90	12	41/ 50 (82%)	0/40 (0%)
Carrick *et al.*, 1990[41]	129	12–36	12/ 59 (20%)	0/70 (0%)
Patchett *et al.*, 1990[41]	51	12	5/ 18 (28%)	0/33 (0%)
Lamouliatte *et al.*, 1991[42]	44	12	15/ 18 (83%)	1/26 (4%)
Graham *et al.*, 1991[43]	100	9	– (95%)	– (0%)
Collins *et al.*, 1991[44]	60	24	11/ 19 (58%)	0/41 (0%)
Logan *et al.*, 1991[45]	20	9	12/ 17 (71%)	0/ 3 (0%)
Fiocca *et al.*, 1991[46]	144	6	55/114 (48%)	3/30 (10%)
Sobala *et al.*, 1992[47]	71	12	25/ 44 (57%)	1/17 (6%)
Coelho *et al.*, 1992[48]	48	18	10/ 19 (53%)	0/19 (6%)
Bayerdörffer *et al.*, 1992[49]	53	12	19/ 31 (61%)	0/22 (0%)
Labenz *et al.*, 1992[50]	48	12	14/ 19 (74%)	1/29 (3%)
Hentschell *et al.*, 1993[29]	104	12	46/ 52 (89%)	1/52 (2%)
Mannes *et al.*, 1993[28]	178	12	(42%)	(6%)
Unge *et al.*, 1993[51]	248	6	(36%)	(6%)
Vigneri *et al.*, 1993[52]	85	12	(63%)	(0%)
Chung *et al.*, 1993[53]	127	12	39/ 62 (63%)	6/65 (9%) (after triple therapy)
Balatsos *et al.*, 1993[54]	65	12	5/ 13 (37%)	0/52 (0%)

with resistant ulcer were randomized to either CBS or CBS plus antibiotics. Of the 39 ulcers which healed, 17 became Hp negative and 21 remained positive. At 12 months none of the first group had relapsed, in contrast with 81% of the Hp-positive patients. As both groups had received CBS it follows that the exposure to this drug could not have been responsible for the difference. All eradicated patients have now been followed for over 3 years and the ulcer relapse rate is still nil[36]. Similar results were recently obtained by Mannes *et al.*[28].

An interesting long-term follow-up study has been presented from Australia[38,56]. The relapse rates for Hp infection and duodenal ulcer were endoscopically studied in 75 relatively resistant ulcer patients, healed and eradicated with triple therapy. After 1 year, 71 of 73 patients remained free of Hp infection and ulcer; the corresponding figures at year 2 were 57/57; at year 3 they were 34/34 and at year 4 they were 15/15. No ulcer recurred in Hp-negative patients who were followed for up to 4 years in spite of continued smoking. Distorted duodenal caps gradually returned to near-normal appearance in several patients by 2 years. I have made similar observations in a

few patients with symptoms compatible with gastric outlet obstruction who became asymptomatic after Hp eradication without balloon dilatation of the narrowed pyloric channel.

GASTRIC ULCER DISEASE

Prevalence of *H. pylori* in gastric ulcer disease

It has been known for over a century that gastric ulceration occurs against a background of chronic gastritis. Only recently has it been appreciated that Hp infection is the cause of this gastritis. Although there is wide variation in the prevalence of Hp infection in gastric ulceration in published series, it has been argued that much of the variation is explained by selection criteria, and if care is taken to eliminate drug-induced ulcers, then the prevalence approaches 100%[57].

Histopathological aspects of gastric ulcer disease

The chronic gastritis which accompanies gastric ulceration is characteristically diffuse chronic pan-gastritis[3,4,58], and frequently exhibits both multifocal glandular atrophy and intestinal metaplasia. The latter features appear to reflect the duration of infection in that their prevalence increases with age[4,59] and longitudinal studies have shown progression from non-atrophic ('superficial') chronic gastritis through atrophy to intestinal metaplasia[60,61]. Various cytopathic mechanisms have been suggested as contributing to atrophy, a direct effect of Hp cytotoxins[17], the high ammonia concentration[62], the liberation of neutrophil proteases or toxic oxygen radicals[63], the formation of crossreacting antibodies[64], and high gastric juice acetaldehyde levels[65]. According to Dixon, intestinal metaplasia may begin as a transient regenerative phenomenon perhaps in the healing of superficial epithelial defects, but with repeated injury (or sustained infection) intestinal metaplasia may become more extensive and permanent[66]. However, atrophy and intestinal metaplasia are not simply long-term sequelae of Hp infection. Other gastric irritants such as bile[67], NSAIDs, a high-salt diet[68], and possibly cigarette smoking[69], may act independently or, more frequently, synergistically with Hp infection to bring about atrophy and intestinal metaplasia. On the other hand, certain dietary factors may exert a protective influence against the development of atrophy; vitamin C[70], vitamin E, β-carotene and trace elements may play some part. Atrophy of glands is accompanied by replacement fibrosis. Although regeneration is a theoretical possibility, this does not occur to any great extent, and a permanent functional deficit remains. Glands lost from the corpus region are partially replaced by metaplastic glands of pyloric type.

The prevalence of Hp in the stomach declines with increasing glandular atrophy. Indeed, the lower rate of detection has led some to deny a role for Hp infection in the causation of non-autoimmune atrophic gastritis[71]. There are two main reasons for the loss of organisms. Firstly, Hp only colonizes

gastric epithelium; thus the organisms are absent from areas of intestinal metaplasia. Secondly, the hypochlorhydric stomach is inimical to Hp. The organism requires an acidic environment in which to thrive, because the ammonia which is released in its vicinity as a consequence of bacterial urease activity remains un-neutralized and accumulates. This may lead to ingress of ammonia into the organism with protonation of intracellular proteins and failure of intermediary metabolism[72]. Thus, death of the bacterium results from a process of auto-destruction, and the failure to demonstrate Hp in the atrophic stomach does not deny a role for infection in the causation of the underlying gastritis.

Having established that Hp is the cause of chronic gastritis, and that it has an important role in the subsequent development of atrophy and intestinal metaplasia, we still have to explain its role in the pathogenesis of ulceration. On the one hand, diminished mucosal resistance can be explained in terms of cytopathic effects on the surface epithelium, increased cellular exfoliation, increased cell turnover and diminished mucus and bicarbonate secretion affecting the mucous barrier, but this sequence of events does not explain why ulcers have a predilection for certain sites in the stomach, or why everyone with chronic gastritis does not develop an ulcer.

The sites at which gastric ulceration occurs are by no means random, as one would expect if it was simply a consequence of the diffuse gastritis. Prepyloric ulcers are epidemiologically linked to duodenal ulceration. Proximal gastric ulcers occur most frequently on the lesser curve at the junction between antral and corpus mucosa. In so far as glandular atrophy and pyloric metaplasia extend cranially with increasing age[73] (and duration of infection?), so the site of ulceration moves up the lesser curve with age[74]. The susceptibility of this junctional area to ulceration, compared to the remainder of the stomach, is not easy to explain. It is, of course, the site where susceptible mucosa is immediately adjacent to acid-secreting mucosa, but this is obviously not confined to the lesser curve. Differences in blood supply or mechanical factors have been invoked, but with little conviction. More persuasive is the suggestion that the junctional site between two epithelia (gastric and metaplastic) is intrinsically less 'stable', and therefore less resistant than a uniform mucosa[75,76]. In fact, the incisura is the site where maximal degrees of atrophy and intestinal metaplasia are found[77]. Besides differences in mucus composition and bicarbonate production, which may lower protection afforded by the mucous barrier, metaplastic and atrophic mucosa may differ from normal in terms of local growth factor production and on the presence of receptors for luminal growth factors such as epidermal growth factor. Diminished growth factor stimulation will adversely affect mucosal regeneration, and thereby exaggerate the effects of injury. Thus, gastric ulceration can be viewed as a consequence of Hp-associated chronic gastritis in which there is either a locally increased susceptibility to injury brought about by severe atrophy and metaplasia in antral-type mucosa, or there is a focally concentrated injury acting in synergy with the gastritis as occurs, for example, with oral NSAID use, when ulceration can occur at any site in the stomach.

Table 3 Gastric ulcer relapse rate in patients with persistent *H. pylori* infection and after eradication of *H. pylori*

	n	*M*	*Hp+*	*Hp−*
Tatsuta *et al.*, 1990[79]	43	3	47	7
Seppäla *et al.*, 1992[80]	204	12	57	0
Graham *et al.*, 1992[81]	26	12	74	0
Bayerdörffer *et al.*, 1992[82]	102	6	33	0

Bacterial characteristics in gastric ulcer disease

If one assumes that surface epithelial degeneration is a necessary preliminary step to ulceration, one might expect that the more ulcerogenic strains would exhibit more surface degeneration. There is some circumstantial evidence for this effect. A higher prevalence of antibodies to a 120 kDa protein of Hp has been found in patients with peptic ulcer when compared to those with functional dyspepsia, and in those patients with more severe degrees of surface epithelial degeneration than those with mild or no epithelial degeneration[19,78]. Nevertheless, when the degree of surface epithelial degeneration was assessed in antral biopsies from Hp-positive gastric ulcer patients and age-matched Hp-positive dyspeptic controls, no difference was found[77], indicating that the presence of coexistent ulceration cannot be explained in terms of differing degrees of 'background' epithelial degeneration elsewhere in the stomach.

Effect of *H. pylori* suppression/eradication on gastric ulcer healing

Only very limited almost anecdotal data are available to evaluate the role of Hp suppression/eradication in the GU healing rate. There are some uncontrolled observations of so-called refractory gastric ulcers which healed only after Hp eradication.

Effect of *H. pylori* eradication on gastric ulcer relapse

Preliminary data are available indicating that GU relapse is significantly reduced after successful Hp eradication, as summarized in Table 3. In Amsterdam we have followed well over 50 well-documented genuine non-drug-associated GU patients after eradication, and have so far not observed any relapse (unpublished observations). It would therefore appear that, for GU also, regression of the mucosal inflammation permanently improves the defence against the acid/peptic attack and thereby eliminates the natural tendency to relapse when ongoing Hp associated inflammation is allowed.

CONCLUDING REMARKS

In any population, a small proportion of individuals will have an intrinsically larger parietal cell mass than 'normal'. Such individuals may develop more

extensive gastric metaplasia in the duodenum. If these individuals acquire Hp infection, inflammation in the stomach remains largely confined to the antrum because of acid protection of the corpus. However, the presence of gastric metaplasia in the bulb permits infection of the duodenal mucosa and an Hp-induced active chronic duodenitis (or perhaps better labelled gastritis in the bulb?) will ensue. Surface epithelial degeneration and increased cell turnover eventually predispose to duodenal erosion and ultimately to ulceration.

Over the years there may be gradual extension of infection and subsequent inflammation from the gastric antrum into the corpus, and atrophy may follow. Acid production diminishes and reduces the burden on the duodenum, thereby gradually decreasing the risk of duodenal ulceration, but may not fall sufficiently to preclude the development of ulceration in the variably atrophic gastric mucosa. Thus there is a gradual switch from a duodenal ulcer to a gastric ulcer phenotype. Alternatively when the Hp infection and the ensuing gastritis occurs during childhood before the parietal cell mass has matured, pangastritis with variable atrophy, predisposing to gastric ulcer disease, becomes possible.

While the removal of the acid-peptic attack can bring about healing of an ulcer, the mucosa will remain inflamed and therefore susceptible to ulceration when acid secretion resumes. The only way to radically alter the natural history of peptic ulcer disease is to restore the gastroduodenal mucosa to health by eliminating Hp infection[83,84]. It is now well established that duodenal ulcer recurrence, and to a lesser extent gastric ulcer recurrence, becomes a rarity after successful eradication. Sufficient evidence has been provided to justify the recommendation to treat all patients with Hp-associated duodenal and gastric ulcer disease with anti-Hp therapy, attempting to eradicate the infection and to restore the health of the gastroduodenal mucosa. Eradication of Hp equals cure of duodenal ulcer disease. Unpublished information would indicate that the beneficial effect of Hp eradication persists as long as no reinfection occurs. The same sequence of events appears to occur for Hp-associated gastric ulcer, but more data are required for unequivocal support of this statement.

Infection with Hp is undoubtedly *the* dominant factor in the multifactorial peptic ulcer diathesis. However, the other contributing factors should not be ignored but rather, one should try to identify how they interact with the organism and initiate the ulcerative process. The interplay of acid attack and mucosal defence is modulated by genetics, gender, blood group, smoking, age, and various physiological considerations which include acid output. These and other considerations probably explain the discrepancy between the high frequency of Hp infection in the population and the comparatively small proportion of individuals who develop a peptic ulcer.

Most agents used in peptic ulcer disease are aimed at reducing acid secretion, and achieve healing by minimizing acid attack. Such treatments, however, have no effect on Hp status and do not remedy the underlying gastroduodenitis. The mucosa therefore remains ill and vulnerable[85,86]. Following cessation of acid suppressive therapy, ulcer relapse is likely. Goodwin[87] has likened the inflamed mucosa to a 'leaking roof', where

temporary dryness (healing) is assured if the rain (acid) is removed but permanent protection can be achieved only by mending the roof through healing of the mucosa. Therefore, therapy which fails to address the role of Hp in the causation of the mucosal inflammation which predisposes to ulceration, is likely to confer only short-term benefit.

Although it has been shown beyond reasonable doubt that Hp infection and associated inflammation is of major or dominant importance in peptic ulcer disease, many questions still need to be explored before full understanding of the entire pathogenic sequence of events is possible. Why the inflammation remains largely limited to the antrum and bulb in duodenal ulcer disease, and what the consequences are of concomitant disturbed gastrin homeostasis, requires further clarification. Why a duodenal ulcer is invariably a localized and periodically recurring phenomenon in the presence of a diffuse and stable infection and inflammation requires explanation. Why eradication of the organism turns out to be so difficult is still an enigma. What the long-term consequences are of Hp eradication requires long-term follow-up. Whether gastric metaplasia in the bulb can regress, and whether the progression of gastric inflammation towards atrophy and intestinal metaplasia can be interfered with, necessitates detailed large-scale prolonged observations. To answer those numerous questions will require carefully designed studies during the years to come.

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ROLE IN GASTRIC CANCER

9 The precancer–cancer sequence

TANYA LEVINE and ASHLEY PRICE

INTRODUCTION

At one end of the pathological spectrum is the acute gastritis associated with the initial confrontation with *Helicobacter pylori*[1,2]; at the other end is gastric cancer. There is growing evidence from the fields of epidemiology and pathology that the two events may be linked. In this chapter the morphological steps in the progression across this spectrum are examined whilst the epidemiological and pathophysiological data are to be found in Chapters 2 and 10. The temporal course, if it exists, is a long one in the majority of cases. Migrant studies suggest that its occurrence might require infection with *Helicobacter pylori* to be established in childhood[3], which is complementary to general concepts that long-standing chronic inflammation is linked to subsequent malignancy at several sites in the body. An appropriate example from the gastrointestinal tract is cancer in colitis, which will be briefly considered later in relation to dysplasia, one of the steps in the precancer–cancer sequence.

BACKGROUND

Prior to the discovery of *Helicobacter pylori* it was appreciated that there were several conditions associated with an increased risk of gastric malignancy. These precancerous conditions include pernicious anaemia, Menetrier's disease, the 'post-gastrectomy' stomach, the adenomatous gastric polyp and late-onset hypogammaglobulinaemia. Most gastric cancer occurs unassociated with such conditions, and it is from this pool that a role for *Helicobacter pylori* has been postulated. Whilst a number of classifications have been proposed for gastric carcinoma the most widely used is that of Lauren[4]. The Lauren classification subdivides gastric adenocarcinoma into two types, 'intestinal' and 'diffuse'. The former is the most prevalent subtype in 'high-risk' gastric carcinoma populations such as Japan, South America and southern Asia, where typically it arises in the gastric antrum of middle-aged men. The latter 'diffuse' form is generally found in a younger population

in whom the gender ratio approaches 1:1, it characteristically arises in the gastric corpus and has an association with blood group A.

Gastric cancer is the fourth leading cause of cancer death world-wide. However, over the past 50 years there has been a steady decline in the incidence of the 'intestinal' type and to a lesser extent of the 'diffuse' form, whilst cancer of the cardia is believed to be on the increase[5,6]. Interestingly, interpretation of data from successive birth cohorts would indicate a similar decline in the prevalence of *H. pylori* infection[7]. Furthermore, with a few exceptions, countries with high rates of gastric cancer also have high rates of *H. pylori* infection and, within these countries, both gastric carcinoma and *H. pylori* colonization are strongly associated with poor socioeconomic conditions (see Chapter 2)[7–9].

The concept of a precancer–cancer sequence in the stomach derives from the longitudinal studies of Finnish workers and Correa *et al.*[10–12]. These workers studied the natural history of chronic gastritis in circumscribed populations over many years. Studies were begun prior to the discovery of *Helicobacter pylori* but demonstrated that the common form of 'intestinal' gastric carcinoma arises on a background of chronic atrophic gastritis and intestinal metaplasia through a multistep progression. This sequential progression suggested by Correa *et al.*[10] of gastritis, then chronic atrophic gastritis, through intestinal metaplasia to dysplasia and finally invasive 'intestinal' adenocarcinoma is lengthy, with the ultimate development of an invasive cancer occurring some 15–20 years after its onset. The morphological aspects of the sequence and its association with *Helicobacter pylori* are examined here. To retain perspective it must be remembered that there are countries in the world with an overall high prevalence of *Helicobacter pylori*, yet that have adjacent regions with contrasting high and low cancer risks[13]. It is therefore unlikely that *Helicobacter pylori* is the sole factor driving the sequence. In the parlance of cancer models it would seem more appropriate to view *Helicobacter pylori* as some form of promoting agent that provides a continuing source of inflammatory damage. On this and an appropriate genetic profile multifactorial initiating agents, such as dietary and other environmental factors, exert their effect[14]. For example, Correa *et al.*[15] are strong supporters for the place of a high-salt diet as such a factor.

Although the precancer–cancer sequence was put forward for the 'intestinal' pattern of gastric cancer if *Helicobacter pylori* does have a role, it is logical to assume it should also be involved in the genesis of the 'diffuse' form (see later).

HELICOBACTER PYLORI AND GASTRITIS

The first step in any association of *Helicobacter pylori* with a precancer–cancer sequence is the initial infection. Because this passes unnoticed in the large majority of instances there is little documented morphological data. The few descriptions of accidental infection or self-administered infection[1,2] indicate there is an initial acute neutrophilic pangastritis which, unless active steps are taken to eradicate the organism, usually progresses over several

months to an antral predominant chronic pangastritis. Important to the underlying mechanism of the precancer–cancer hypothesis is that very few individuals eliminate the organism spontaneously, ensuring there is long-standing subclinical inflammation in the majority of the infected populations. This is an appropriate background for a gradual transition to neoplastic proliferation in a selected group.

Although data on the initial infection are scant, there are abundant data linking *Helicobacter pylori* to chronic gastritis, the second step in the progression. There are three broad topographical categories of chronic gastritis: chronic gastritis of the antrum, chronic gastritis of the corpus and chronic pangastritis which involves both compartments, though usually antral disease predominates[16–19]. Inflammation restricted mainly to the antrum is seen in those patients with an increased risk of duodenal ulcer disease. Of the three patterns it has the strongest associations with *Helicobacter pylori*. However this pattern of inflammation tends not to progress along the precancer–cancer pathway[16,18,19].

Gastritis limited to the corpus is uncommon. When accompanied by atrophy most patients are found to have pernicious anaemia and are reported to carry an increased risk of gastric carcinoma[20]. The risk is likely to be part genetically determined and part related to atrophy, an aspect that is considered later. *Helicobacter pylori* is seldom seen on the mucosa in patients with pernicious anaemia[21] in whom atrophy is limited to the corpus. However, antibodies to *Helicobacter pylori* have been detected in some[22] with corpus atrophy, suggesting that the organisms might have initiated the corpus damage in a genetically defined patient cohort who go on to develop clinical pernicious anaemia. Furthermore crossreacting antibodies between the organism and the gastric mucosa have been identified[23].

The commonest topography of chronic gastritis associated with *Helicobacter pylori* is of a pangastritis. In most this remains limited to low-grade inflammation predominant in the antrum with less severe and slightly patchy corpus involvement. It is from this morphological pattern that patients with gastric ulceration and gastric carcinoma emerge[19,24].

HELICOBACTER PYLORI AND ATROPHY

The natural history of chronic gastritis occurs over a 10–20-year period. The studies from Finnish and Estonian populations, and the longitudinal studies from Colombia and Peru[10–12,25], show that in a proportion of patients atrophy becomes part of the natural progression. Atrophy is defined as loss of the specialized mucosal glands, be they of corpus or antral type. Although this normally occurs in conjunction with the development of intestinal metaplasia (see below) this is not necessarily the case[26]. In northern European studies approximately 3% of adults per annum develop gastritis, and 3% of this pool are likely to progress to an atrophic picture. Several studies correlate increasing atrophy with age, but this turns out to be *Helicobacter pylori*-related[28,29]. The onset of atrophy increases the relative risk of developing a gastric cancer, the risk varying with the compartment of the stomach

involved. The risk has been calculated as three to four times for the severe corpus atrophy seen in patients with pernicious anaemia, up to 18 times when there is severe antral atrophy and when both compartments show severe atrophy, which is usually patchy, the overall risk is a multiplicant of both, reaching a relative risk of up to 90 times[27,30]. In addition to epithelial neoplasia it is known that the hypergastrinaemia associated with atrophy of the corpus promotes enterochromaffin-like cell hyperplasia and occasional carcinoid tumours in some patients[31]. Pure achlorhydria unassociated with atrophy, as seen in prolonged administration of proton-pump antagonists, does not seem to have this effect[32].

With *Helicobacter pylori* established as a strong associate of two of the three main topographical patterns of gastritis, chronic antral gastritis and chronic pangastritis, it is the latter which has the greatest potential for progression to atrophy and a central role in the precancer–cancer sequence. Correa[33] proposes the name 'multifocal atrophic gastritis' for this pattern, and believes it is a distinct nosological entity. Epidemiological and cross-sectional studies show wide variation in the cancer risk between countries and, as previously emphasized, sometimes between areas within the same country with similar prevalence figures for *Helicobacter pylori* colonization[13]. These data emphasize the multifactorial nature of gastric carcinogenesis, making it difficult to quantify the exact role of *Helicobacter pylori* in the sequence from initial infection through a particular topography of gastritis to atrophy and on to carcinoma. This difficulty is compounded by the knowledge that, at least for the intestinal pattern of carcinoma on which the proposals for the sequence are based, the risk of cancer is highest in the atrophic stomach, yet the atrophic hypochlorhydric stomach is hostile to *Helicobacter pylori* colonization[34]. This contrasts with the initiation of infection in which a period of hypochlorhydria seems to be essential for the bacteria to become established. The inverse relation of colonization success to atrophy suggests that *Helicobacter pylori* is likely to be involved early in the precancer–cancer sequence. This fits well with the observation that in areas and populations with a high incidence of gastric cancer a high incidence of *Helicobacter pylori* infection is found in childhood[7,25]. A key unanswered question is at which stage, in the high-risk environment, is the sequence reversible if the organism is eradicated?

HELICOBACTER PYLORI AND INTESTINAL METAPLASIA

Intestinal metaplasia was associated with the intestinal variant of gastric carcinoma well before the era of *Helicobacter pylori*[35]. It is somewhat artificial to consider it in isolation from atrophy as the two, with some exceptions, occur alongside each other. Indeed, because metaplasia is more easily appreciated down the microscope than the milder forms of atrophy, Yardley has proposed that chronic pangastritis with atrophy (the multifocal atrophic gastritis of Correa[33]) be termed metaplastic atrophic gastritis[36].

There are three main varieties of intestinal metaplasia[37] but probably only one type seems to be implicated in the gastric cancer sequence[38]. In type I

areas of mucosa are replaced by non-secretory absorptive cells with a brush border interspersed with goblet cells secreting sialomucin (complete type). In type II the goblet cells are interspersed between neutral or sialomucin-secreting cells. In type III the goblet cells are found between mucin-secreting cells, at least some of which are secreting sulphomucin. There is some evidence that types I and II can be short-lived changes seen against a background of inflammation and capable of regression[39,40]. Type III is the pattern most often implicated in the precancer–cancer sequence and thought to be irreversible. When present surrounding inflammation is more muted and occasionally dysplasia may be present. One morphometric study claims that type III metaplasia already has some of the characteristics of dysplasia[41]. The subtypes are seldom seen in a pure form and, whilst types I and II are relatively common, type III metaplasia is rare. This has meant that typing metaplasia for cancer surveillance purposes has resulted in conflicting results about its significance[42]. Metaplasia probably commences in the region of the incisura, from where it spreads to involve increasing amounts of the mucosa. It is interesting that it is this site, the junction of the acid- and non-acid-bearing part of the gastric mucosa, at which gastric ulceration is most common. This junction is believed to progress proximally with age[43].

As with atrophy *Helicobacter pylori* fails to colonize metaplastic epithelium. Despite this there are now many studies correlating the presence of metaplasia with the presence of *Helicobacter pylori* in the adjacent non-involved mucosa[44,45]. In one study[44] of 533 individuals attending a gastrointestinal endoscopy clinic overall 25.3% had intestinal metaplasia. It was more common in *Helicobacter pylori*-positive than -negative individuals, 33.9% vs 15.3%. Both *Helicobacter pylori* and intestinal metaplasia increased with age. The same group[46] showed the organism had a negative correlation with type III metaplasia, and suggested that at that stage the hostile environment extended beyond the confines of the metaplastic cells. Other studies also support a relationship between *Helicobacter pylori* and metaplasia. Sobala *et al.*[40] demonstrated a synergistic effect between bile reflux and *Helicobacter pylori* on the induction of intestinal metaplasia.

The role of *Helicobacter pylori* and metaplasia is highlighted in a particular case report of a British cancer family[47]. Four siblings are documented, two of whom developed intestinal pattern gastric carcinoma in the fourth decade. A third underwent a gastric antrectomy for dysplasia, and the fourth developed severe atrophic gastritis and intestinal metaplasia at an early age. Study of their eight children showed that five (63%) between the ages of 10 and 26 years developed *Helicobacter pylori*-associated chronic atrophic gastritis, and in three of these (60%) intestinal metaplasia was present in the antrum. By contrast one would expect, in a European population under the age of 30 years, less than 4% to have metaplasia. From this the authors postulated that, in a genetically susceptible family group such as this, *Helicobacter pylori* promoted the development of intestinal metaplasia at an early age, and that the subsequent sequelae were a consequence of *Helicobacter pylori* triggering accelerated proliferation within the gastric stem cells.

In contrast to the proposed role in the precancer–cancer sequence of

intestinal gastric cancer intestinal metaplasia has no role in the development of the diffuse pattern of carcinoma.

HELICOBACTER PYLORI AND DYSPLASIA

Dysplasia is well established as the penultimate morphological stage in the precancer–cancer sequence at many sites in the body, not just the stomach. In the stomach, when high-grade dysplasia is detected, many patients will already be found to harbour a carcinoma or develop one within a year[48,49]. For these reasons the reliable and confident discovery of high-grade dysplasia indicates that immediate clinical consideration be given to a gastrectomy. The implications of low-grade dysplasia are less clear-cut[48–50] with a failure to progress, or regression, being documented in up to 50% of some series. Contrasting with intestinal metaplasia dysplasia is a recognized step in the development of diffuse carcinoma[51].

There are very few specific data on the association of dysplasia with *Helicobacter pylori.* Isolated high-grade dysplasia is a relatively rare lesion, the majority are associated with a pre-existing cancer or seen alongside metaplastic changes. Thus in the dynamics of the precancer–cancer sequence model it is probably impossible to separate out any special relationship with *Helicobacter pylori.* Furthermore with the known reports of regression of some cases of low-grade dysplasia, even though *Helicobacter pylori* status was not commented upon, it seems unlikely still to be the driving mechanism at this stage of the cancer sequence. However, in one series of seven patients with dysplasia it is claimed that it regressed in six after *Helicobacter pylori* was eliminated with bismuth therapy[25]. Because there is so much observer variation in the documentation of dysplasia such observations need confirmation. Nevertheless this isolated report requires renewed attention now that regression of gastric lymphoma has been documented following *Helicobacter pylori* eradication (see below)[52].

If dysplasia in the colon is compared with that in the stomach, in the former it commonly takes the form of a polyp (adenoma) in the precancer–cancer sequence. In the stomach adenomatous polyps are rare and dysplasia in the precancer–cancer sequence is flat. By contrast flat dysplasia in the colon is rare, and seen only as a complication of ulcerative colitis. Thus at both sites, the stomach and the colon, flat dysplasia is the sequela of long-standing chronic inflammation due to *Helicobacter pylori* infection in the former and, perhaps, an as yet unidentified infective trigger in the latter.

HELICOBACTER PYLORI AND CARCINOMA

The morphological association of *Helicobacter pylori* with established gastric cancer is between 19% and 80% with a mean around 50%[53]. It has been pointed out that the stages of intestinal metaplasia and atrophy in the precancer–cancer sequence result in a gastric microenvironment hostile to *Helicobacter pylori.* Such a wide range in results is therefore understandable

as the negative sampling of metaplastic and atrophic areas will distort the figures. Furthermore, many of the series have been retrospective and based on diagnostic material from which limited sections were taken, and at a time when identification of *Helicobacter pylori* was not a prime interest. If only malignant tissue is examined the tissue prevalence is around 10%[54]. Additional variation occurs because up to 25% of gastric cancers arise in *Helicobacter pylori*-negative individuals with severe corpus atrophy[55]. Discrepancies between serological and morphological methods of detection will also add to the variation in results. In the study by Sipponen *et al.*[55] the overall agreement between these two methods was 72% with the presence of atrophy being the most discriminating factor when organisms were not found. The morphological data are supported by a series of prospective serological studies showing an increased risk of gastric cancer in infected individuals of between 2.8 and 6.0 times.

Whilst the model for the precancer–cancer sequence was constructed for the intestinal pattern of gastric carcinoma, as mentioned previously one might expect the effect of *Helicobacter pylori* also to influence the development of diffuse carcinoma. Although there are some conflicting data the two forms do have a similar prevalence of associated *Helicobacter pylori*. Sipponen *et al.*[55] showed that 70% of gastric cancer patients were *Helicobacter pylori*-positive with no significant difference between the two patterns. Loffeld *et al.*[56] found a *Helicobacter pylori* prevalence of 60% and 45% in intestinal and diffuse patterns respectively; this too was not significant and the same was found by Talley *et al.*[57]. In the studies of one other group conflicting results of differences between the two patterns have been reported[58,59].

In diffuse gastric carcinoma the malignancy arises on the background of chronic gastritis via dysplasia, but in the absence of atrophy and metaplasia[51]. Sipponen and Seppala[60] suggest that *Helicobacter pylori* still has a role in the initial stages, but other factors come into play at an earlier stage in the precancer–cancer sequence, prior to the advent of atrophy and metaplasia. Supporting this concept diffuse gastric carcinoma arises in a younger population than its intestinal counterpart.

Although the data reviewed here support the role of *Helicobacter pylori* in the promotion of gastric cancer there are controversial findings from Africa[61], where high colonization rates are found but cancer is uncommon. Whether this is fact or merely poor statistical documentation awaits clarification.

CANCER OF THE CARDIA

Although the incidence of antral and corpus gastric cancer is falling worldwide, cancer of the cardia may be on the increase[62]. There are, however, very few good data on *Helicobacter pylori* colonization of the cardia, let alone any relationship between this and cancer of the cardia. One study[6], looking at the population of Oxfordshire between 1960–64 and the period 1984–88, suggests there was an increased association of the organism with cardiac tumours between the two periods from 1.8% to 32.5%. In the same

periods the association with antral tumours fell from 80% to 40%. This was a retrospective study, and it is difficult to accommodate such data into any coherent theory about the changing pattern of gastric cancer, the precancer–cancer sequence and *Helicobacter pylori* associations. Perhaps more informative are the prospective studies using banked serum. Two such studies[57,59] failed to show any association of *Helicobacter pylori* with tumours of the cardio-oesophageal junction and cardia.

HELICOBACTER PYLORI AND MALIGNANT (MALT) LYMPHOMA

The normal gastric mucosa is devoid of lymphoid follicles and several studies exist showing a strong association between the appearance of mucosal lymphoid tissue and *Helicobacter pylori*[63]. In children[64] the appearance of a nodular mucosa due to lymphoid follicles is almost pathognomonic of *Helicobacter pylori* colonization. The lymphoid tissue is of the mucosa-associated type (MALT)[65] and a strong association has been shown between *Helicobacter pylori* and cases of malignant gastric lymphoma of MALT type[66]. It has also been demonstrated that specific strains of *Helicobacter pylori* provide the antigenic drive, via T-cells, to maintain the growth of B cell tumour lines *in-vitro*[67]. In parallel with this, in a group of five cases of MALT lymphoma with limited tumour bulk, the eradication of the organism resulted in resolution of the tumour[52]. In this respect there is a strong resemblance to the behaviour of alpha-chain disease in which, at a certain stage, antibiotics produce a similar tumour resolution. If these findings are corroborated then they provide the strongest evidence to date of an association between malignant tumours and *Helicobacter pylori.*

CONCLUSIONS

The broad morphological aspects of the precancer–cancer sequence and *Helicobacter pylori* outlined here need to be placed in the perspective of the undoubted multifactorial aetiology of gastric carcinogenesis. This includes host factors, for example the equal sex ratio for infection yet the male dominance for cancer, environmental factors and virulence factors of the organism. Many of these issues are addressed in other chapters. At a cellular level, besides the effect of *Helicobacter pylori* on increasing epithelial cell proliferation, are other products of the inflammatory and host immunological response such as cytokines and free radicals, some of which might be directly mutagenic over the prolonged time-course of infection. The environmental aspects include the data from nutritional epidemiology implicating a role for diets high in salt and low in fresh vegetables and fruit. To this must be added the modulating role of the host's genetic profile and the physiological mucosal and DNA repair mechanisms. Whether *Helicobacter pylori* really does have a pivotal role amongst all these variables ultimately depends on showing that, in areas with a high incidence of gastric cancer and *Helicobacter pylori* colonization, eradicating the organism eradicates or significantly reduces the

incidence of gastric malignancy. One example of such challenging data already alluded to is the regression of gastric lymphoma of MALT-type following *Helicobacter pylori* eradication therapy.

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10
Biochemical aspects

GEORGE SOBALA

INTRODUCTION

The epidemiological evidence for a link between *H. pylori* infection and gastric carcinogenesis has been presented elsewhere in this volume. In this chapter I will consider the possible biochemical mechanisms which underlie this link.

The Laurens classification subdivides gastric cancer into two types, intestinal and diffuse. The intestinal type is predominant and its incidence varies widely throughout the world: it has thus also been termed the 'epidemic' type. The diffuse type has a more homogeneous geographical distribution. Most hypotheses concerning mechanisms of gastric carcinogenesis relate to the intestinal type.

Correa and colleagues have proposed a hypothesis, since updated on several occasions[1], by which intestinal-type gastric cancer is the last of a sequence of changes in the gastric mucosa. It is suggested that the first step is the conversion of normal to gastritic mucosa. In the original hypothesis, formulated before the discovery of *H. pylori*, the causative agent for this transformation was unknown. The hypothesis further suggests that some patients with chronic gastritis develop glandular atrophy with a reduction in gastric acid output. This allows bacterial overgrowth to occur. Some bacteria are capable of reducing dietary nitrate to nitrite, which can form a number of highly reactive chemical species such as N_2O_3 and the nitrosyl ion NO^+. These are nitrosating agents, i.e. they can react in their turn with amines, amides and other molecules in aqueous solution to form the corresponding *N*-nitrosocompounds (NOC), many of which are mutagens. According to this hypothesis, these then act on the gastric epithelium to cause a sequence of mutations resulting in first intestinal metaplasia, then dysplasia and finally carcinoma.

The evidence supporting most steps of the hypothesis is strong. Conditions giving rise to hypochlorhydria, such as pernicious anaemia, gastric surgery and hypogammaglobulinaemia, are all associated with an increased risk of cancer. Bacterial overgrowth does occur and gastric juice nitrite levels are high in the presence of high juice pH and of precursor conditions such as

atrophy and intestinal metaplasia. Chemical nitrosation has been shown to occur both *in vitro* and *in vivo* in humans. The major problem with the hypothesis concerns the *N*-nitrosocompounds. There are too many of these to measure individually, and it is not known which are likely to be clinically relevant. Many attempts have therefore been made to measure 'total *N*-nitrosocompounds' as a group, but the various methods have been criticized either for being too selective or for lacking specificity. Unsurprisingly, results have been contradictory.

The *N*-nitrosocompound step of the hypothesis also seemed to run into a physicochemical barrier. The reaction of nitrite-derived species with precursor amines and amides is acid-catalysed, and the reaction rate is negligible at neutral pH, yet nitrite concentrations are only high at such pH. More recent work, however, has demonstrated that many species of bacteria are capable of catalysing nitrosating reactions at neutral pH[2], and such bacteria have been detected in achlorhydric gastric juice.

How does *H. pylori* fit into this hypothesis? It is the cause of most cases of chronic gastritis, and so it neatly explains the first transformation of normal to gastritic mucosa. The details of this are considered in a separate chapter in this volume. Does it play any further role in the carcinogenic process? The participation in the *initial* step of the hypothesis should not be confused with the term '*initiator*' as used in carcinogenesis. Initiation of carcinogenesis represents either mutation of normal growth control genes, or alteration of their expression to produce an active oncogene. A promoter enhances expression of a preneoplastic or neoplastic clone of cells, or inhibits anti-cancer defence mechanisms. Most promoters require continuous application over long periods and act by stimulating cell proliferation.

I will therefore consider the evidence as to whether *H. pylori* infection produces classical chemical initiators or promoters of carcinogenesis.

NITRITE

H. pylori is indeed a form of 'gastric bacterial overgrowth'. However, it does not reduce nitrate, and it is thus not a *direct* source of nitrosating agents.

GASTRIC JUICE *N*-NITROSOCOMPOUNDS

Accepting the aforementioned problems with the assays, there is also no evidence that gastric juice *N*-nitrosocompounds are generally elevated in patients with *H. pylori* chronic gastritis which we were able to confirm using the latest assay methodology of the International Agency on Research on Cancer, Lyon, France[3]. This does not exclude the possibility that a subgroup of infected patients — e.g. those developing atrophic gastritis and hypochlorhydria — have enhanced synthesis of specific *N*-nitrosocompounds. Furthermore, as will be discussed later, *H. pylori* infection affects gastric juice levels of the potent antioxidant, ascorbic acid, and by doing so may alter the nitrosation potential of gastric juice.

MUCOSAL *N*-NITROSOCOMPOUNDS

Within the past decade there has been made the startling discovery that a gas, nitric oxide, is involved in a wide diversity of functions within the body[4]. It is produced by the enzyme nitric oxide synthetase, which has two forms. The first form is constitutively expressed on vascular endothelium, and the nitric oxide there produced equates with the entity previously known as endothelial-relaxing factor. The second form of the enzyme produces quantities of nitric oxide several orders of magnitude greater, but is not constitutive — it is inducible and expressed by activated macrophages and neutrophils (and other cells). Here, nitric oxide may have an antibacterial role. Nitric oxide is not itself a nitrosating agent but it can react with oxygen in aqueous solution to produce a variety of potent ones. *N*-nitrosocompound production has been demonstrated by inflammatory extravasated activated neutrophils *in vitro*[5], and there is some evidence that intramucosal nitrosation reactions can occur *in vivo* in human antral mucosa[6]. There is as yet no work in this area relating directly to *H. pylori* or chronic gastritis, but as mucosal neutrophil infiltration is often a feature of chronic gastritis the possibility that *H. pylori* infection generates carcinogens directly within the mucosa definitely deserves investigation.

REACTIVE OXYGEN SPECIES

Activated inflammatory cells also produce reactive oxygen metabolites. These are highly reactive chemical species, many of which are also free radicals (possessing singlet electrons). Depending on circumstances, free radicals may act either as initiators by activating procarcinogens or by directly damaging DNA, or as promoters by causing cell damage (e.g. by damaging membranes by lipid peroxidation) and thus stimulating regeneration[7]. Using a non-specific chemiluminescence technique, one group has found evidence for increased intramucosal reactive oxygen metabolite production both in duodenal and antral mucosa[8,9] of patients infected with *H. pylori*. The relevance of this to gastric carcinogenesis remains to be determined.

AMMONIA

H. pylori expresses a powerful urease enzyme, which catalyses the conversion of urea to ammonia. Individuals with *H. pylori* infection have detectably higher ammonia concentrations in gastric juice[10]. Ammonia may be directly toxic to epithelial cells. As has already been stated, any agent that causes cell death or damage may act as a promoter of carcinogenesis by stimulating regeneration. One group has done a series of studies demonstrating that concentrations of ammonia comparable to those found in juice of infected individuals can cause gastric atrophy in rats[11], increase epithelial cell proliferation[12], and act as a promoter in the methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) rat model of gastric cancer[13].

CYTOTOXIN

As is discussed elsewhere in this book, most but not all strains of *H. pylori* express an 87 kDa protein which is cytotoxic to a variety of epithelial cell lines *in vitro*. It is not clear whether this protein is cytotoxic *in vivo*, but there is evidence suggesting that individuals infected with cytotoxin-positive strains show more epithelial surface degeneration of their gastric mucosa and have a dense mucosal neutrophil infiltrate. Individuals with atrophic gastritis are more likely to carry cytotoxic strains than are subjects with non-atrophic *H. pylori* gastritis[14], and *H. pylori* ELISA-positive patients with gastric cancer are more likely to have antibodies to a 120 kDa protein which acts as a 'marker' for presence of the cytotoxin than are *H. pylori* ELISA-positive subjects with non-ulcer dyspepsia[15]. It is thus possible that the *H. pylori* cytotoxin is acting as a promoter by causing cell damage and stimulating regeneration. Given the association of gastric cancer with tobacco smoking, it is an intriguing observation that nicotine potentiates the effect of the cytoxin[16]. *H. pylori* infection alters mucosal levels of cytokines such as IL-1, IL-6 and TNF-α (as described elsewhere in this volume) and this is an alternative method by which it may affect cell proliferation.

BILE

Duodenogastric reflux of bile salts has been implicated in gastric carcinogenesis. Bile salts may be directly toxic to gastric epithelium, or may be substrates for the production of carcinogenic *N*-nitrosocompounds. The increased risk of gastric cancer following peptic ulcer surgery[17] may be attributable to the promotion of bile reflux. We have demonstrated that gastric juice bile acid concentrations are predictive of atrophy and intestinal metaplasia even in the unoperated stomach, and that there may be synergism between the presence of *H. pylori* and bile reflux[18]. This is illustrated in Fig. 1.

SALT

There is epidemiological evidence suggesting that salt consumption is a risk factor for gastric cancer[19]. Excess dietary salt induces gastric atrophy in mice, and Correa's group have done work demonstrating an association between a measure of sodium intake and the occurrence of atrophic gastritis in an area with a high risk of stomach cancer[20]. Further work is needed to explore how *H. pylori*, bile reflux and salt intake may interact to cause mucosal atrophy.

ASCORBIC ACID

Another potential mechanism by which *H. pylori* may increase the risk of gastric cancer relates to its effect on concentrations of ascorbic acid in gastric juice. We have performed a series of studies in this area over the past 5 years.

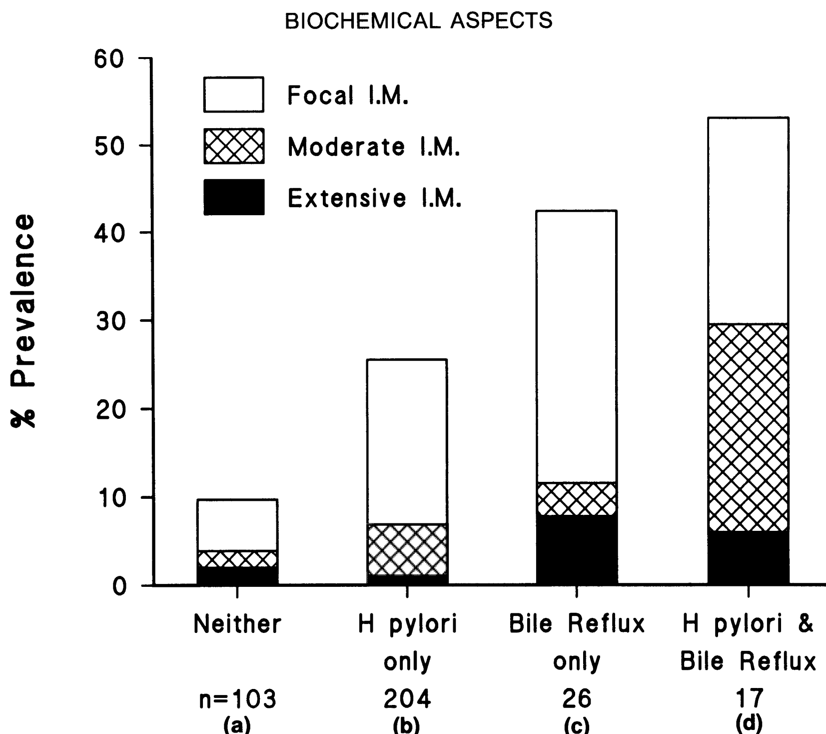

Fig. 1 Prevalence of intestinal metaplasia (IM) in four groups of patients: (**a**) no *H. pylori*, gastric juice bile acid concentration $\leq$ 1 mmol/l, (**b**) *H. pylori* only, (**c**) bile acids > 1 mmol/l only, (**d**) *H. pylori* + bile acids > 1 mmol/l. (Data from ref. 18)

Vitamin C and ascorbic acid are not synonymous. The oxidized form of ascorbic acid, dehydroascorbic acid, can also prevent scurvy and thus both compounds can be considered to be 'vitamin C'. However, only ascorbic acid has antioxidant properties. Dehydroascorbic acid can be enzymatically reduced back to ascorbic acid, but this does not occur in the gastric juice. Most simple laboratory methods for vitamin C assay measure both reduced and oxidized forms together. Only more recently have techniques been developed to separately assay ascorbic acid in body fluids.

Ascorbic acid can prevent the formation of *N*-nitrosocompounds by reacting with nitrite-derived nitrosating species to produce nitric oxide. This gas is not a nitrosating agent, and will equilibrate with the gaseous form and gradually be lost from solution. However, in the presence of oxygen nitric oxide will react to form other oxides of nitrogen, which in turn can again generate nitrosating agents. The reaction of ascorbic acid with nitrosating agents is thus not stochiometrically fixed, but depends on oxygen tension and factors affecting the mass transfer of nitric oxide from solution[21]. Despite the complexities of this set of reactions, ascorbic acid has been shown to prevent *N*-nitroso compound formation *in vitro* and *in vivo*. In rats and mice, administration of ascorbic acid reduces tumour formation in response to dietary nitrite and amines (for review see Kyrtopoulos[22]).

Some historical studies, followed up by work in Leeds[23], made the

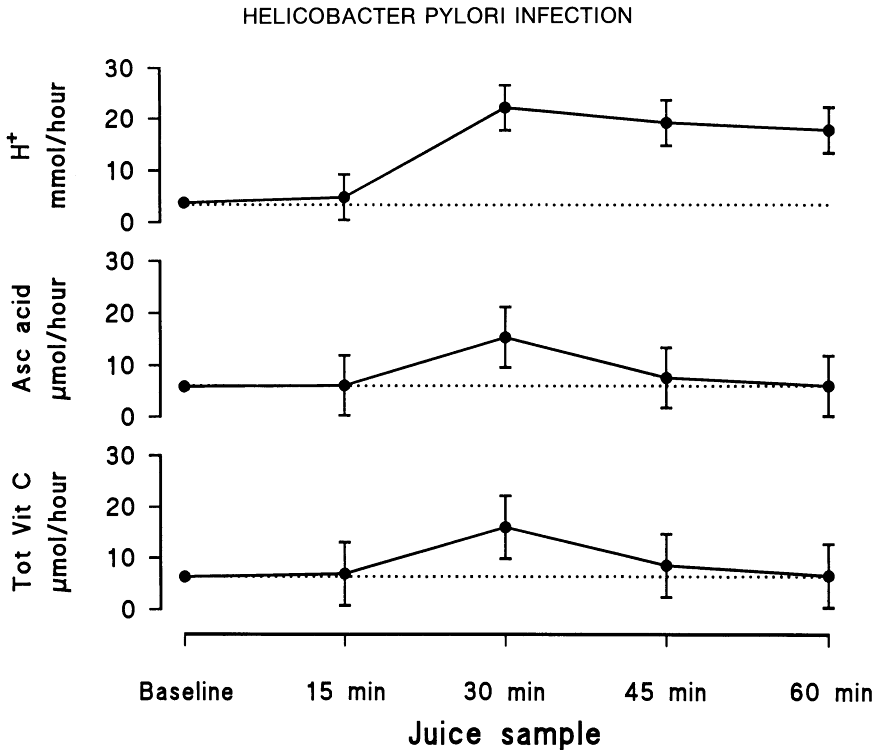

Fig. 2 Effect of pentagastrin on hydrogen ion and ascorbic acid secretion into gastric juice in six *H. pylori* ELISA-negative volunteers. Error bars represent 95% confidence intervals for difference from baseline (dotted lines)

observation that some individuals had high levels of total vitamin C in fasting gastric juice. We then utilized a method that allowed separate determination of ascorbic acid, and explored the relationship between concentrations in gastric juice and gastric pathology. We found that individuals with normal gastric mucosa had high gastric juice ascorbic acid and total vitamin C concentrations, often greatly exceeding those in plasma. This suggests that there is a secretory mechanism in the stomach for ascorbic acid.

We went on to assess the effect of a standard dose of pentagastrin on ascorbic acid secretion into gastric juice in six young volunteers who were negative by ELISA for antibodies to *H. pylori*, and who would thus be expected to have normal gastric mucosa. The results shown in Fig. 2 indicate a transient increase in ascorbic acid output approximately 30 min after pentagastrin injection. This contrasts with a sustained increase in hydrogen ion secretion, and is similar to what is observed with intrinsic factor secretion. An interpretation is that this reflects depletion of an intracellular store, which then requires time to reaccumulate.

Subjects with *H. pylori*-associated chronic gastritis had significantly lower gastric juice vitamin C and ascorbic acid levels than normal subjects (Fig. 3)[3,24,25]; such patients had no overall plasma to juice concentration gradient.

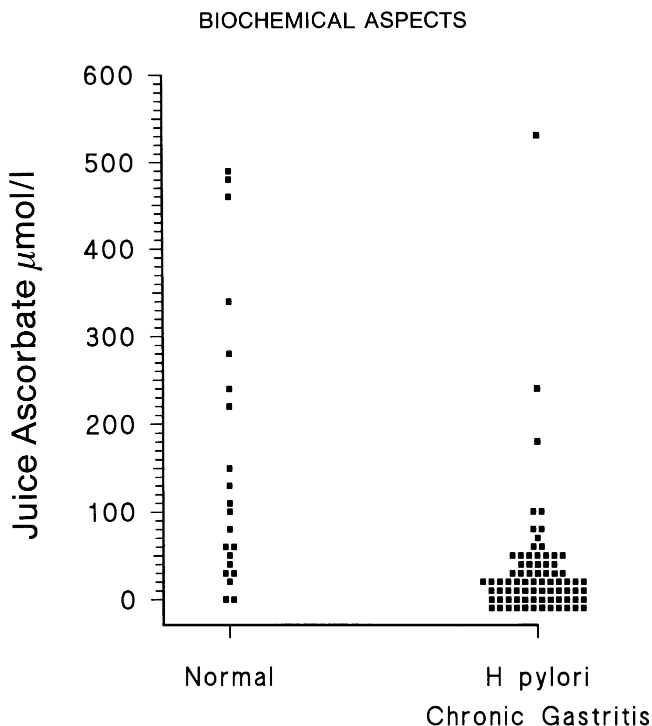

Fig. 3 Gastric juice ascorbic acid concentrations in subjects with normal gastric mucosa v. subjects with *H. pylori* chronic gastritis[3,24]

These results are not explicable by possible dietary changes caused by infection, as *H. pylori*-infected subjects had neither lower plasma vitamin C levels nor lower dietary intake as estimated by questionnaire. The oxidation state of vitamin C depended largely on gastric juice pH; in neutral juice most was present as dehydroascorbic acid.

These data suggest that the onset of *H. pylori* infection in an individual should be accompanied by a fall in gastric juice ascorbic acid concentrations. It seemed difficult to test this hypothesis until I unexpectedly developed symptoms of a similar nature to those reported by Arthur Morris after his voluntary ingestion of *H. pylori*. Infection with *H. pylori* was confirmed by a change in isotope carbon-labelled urea breath testing from negative to positive, seroconversion on ELISA to *H. pylori*, positive culture of the organism and the development of initially acute neutrophilic and then chronic *H. pylori*-associated gastritis on histology. By chance I had acted as my own subject and undergone aspiration of gastric juice via nasogastric tube before and after intravenous injection of 500 mg of ascorbic acid, 170 days before the onset of symptoms. The same protocol was thus repeated 37 days and 161 days after infection[26].

Before infection ascorbic acid was present in gastric juice and levels rose sharply after i.v. injection; 37 days after infection gastric juice was of neutral pH and ascorbic acid levels were effectively zero before and after intravenous supplementation. By 161 days gastric acid output had returned but gastric

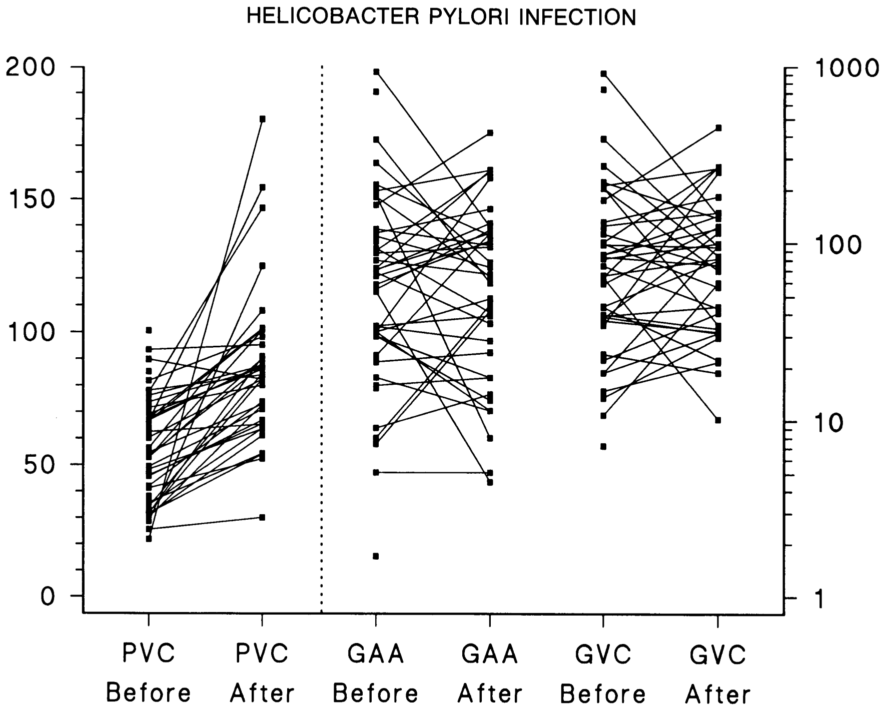

Fig. 4 One week oral supplementation with ascorbic acid in Venezuelan subjects with persistent *H. pylori* infection increases plasma but not gastric juice vitamin C concentrations[29]. PVC = plasma total vitamin C; GAA = gastric juice ascorbic acid; GVC = gastric juice total vitamin C

juice ascorbic acid levels remained low.

Eradication of *H. pylori* restores impaired ascorbic acid secretion into gastric juice. In subjects in whom eradication was successful pre-treatment gastric juice ascorbate concentrations rose by a median 30 μmol/l (95% CI + 10 to +76) from a bseline of 12 μmol/l, and the ratio of gastric juice to plasma total vitamin C rose by a median of 0.78 (+0.29 to +3.10) from a baseline of 1.25. Plasma vitamin C levels did not alter, but the greatest increases in gastric juice levels occurred in subjects with higher pre-treatment plasma levels. There were no significant changes in any of the above parameters in individuals in whom eradication was unsuccessful[27]. These findings with respect to the effect of *H. pylori* eradication have been confirmed[28].

In collaboration with the IARC, Lyon, we have assessed whether oral supplementation with vitamin C can raise low gastric juice ascorbic acid levels in a group of 41 subjects from an area with a high incidence of gastric cancer in Venezuela. Over 90% were infected with *H. pylori* as assessed by a variety of techniques. They received 1 week's treatment with oral vitamin supplements including 500–750 mg of vitamin C. They were then re-endoscoped and gastric juice was obtained for ascorbic acid assay. Although oral supplementation led to a sharp rise in plasma vitamin C levels, there was no change in gastric juice ascorbic acid levels (Fig. 4)[29]. Thus

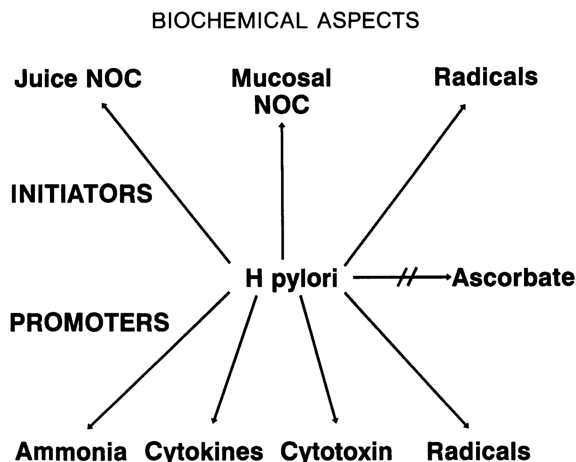

Fig. 5 Summary of potential effects of *H. pylori* on induction and promotion of gastric cancer

enhancement of whole-body vitamin C stores did not increase intragastric secretion of ascorbic acid in subjects with persistent *H. pylori*-associated gastritis.

What effect could the impairment of gastric juice ascorbic acid levels by *H. pylori* infection have on nitrosation potential? With respect to 'classical' acid-catalysed nitrosation, the reaction kinetics have been well described by Licht *et al.*[21], and their mathematical model has been validated *in vivo*. I have adapted their model to estimate the effects of *H. pylori* infection on nitrosoproline formation as mediated by gastric juice ascorbic acid levels.

The kinetic equations were rewritten as ordinary differential equations and solved using the typical gastric juice concentrations of ascorbic acid as found in normal subjects and in subjects with *H. pylori* chronic gastritis as starting conditions. The models were also run separately with thiocyanate concentrations typical of smokers and non-smokers as thiocyanate is a potent catalyst of nitrosation. The results demonstrated a considerable increase in acid-catalysed nitrosation associated with the low gastric juice ascorbic acid concentrations of *H. pylori* chronic gastritis.

The results also emphasize that these reactions become negligible above pH 3.0. However, although ascorbic acid does not react with nitrite at neutral pH, Leach *et al.* have shown that it is a potent inhibitor of bacterial enzyme-mediated *N*-nitrosation, achieving 85% inhibition at a molar ratio of ascorbate to nitrite of only 0.04[30]. Thus the effects of *H. pylori* on gastric juice ascorbic acid concentrations are sufficiently large to have substantial theoretical effects on both acid and bacterial catalysed *N*-nitrosation.

CONCLUSION

H. pylori infection brings about a multiplicity of changes in the chemistry of both gastric juice and the gastric mucosa, and there are many potential mechanisms to explain the observed association with gastric cancer (Fig. 5).

The precise importance of each of these to gastric carcinogenesis remains to be determined.

Acknowledgements

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11
Histological diagnosis

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INTRODUCTION

Helicobacter pylori is one of a small select group of organisms that can be confidently recognized by histology. The histopathologist is uniquely placed both to identify infection and to assess the disease processes associated with it.

NON-SPECIFIC STAINING METHODS

Warren's demonstration of 'curved bacilli on gastric epithelium' was facilitated by a silver staining technique, the Warthin–Starry stain, long used for the detection of spirochaetes in tissue sections[1]. Various alternatives, which claim greater sensitivity, have been introduced, but the methods are capricious, time-consuming and expensive. More recently, a commercial staining kit based on the Steiner technique[2] has been introduced. The method involves microwave heating the sections in uranyl nitrate solution and achieves more consistent results, although background precipitation can still be a problem. Several of the reagents used in these silver impregnation methods are irritant, or potential carcinogens. With these drawbacks in mind, simplified histochemical stains have been introduced, the most widely employed being modifications of the Giemsa stain[3] in which a longer staining time and absence of differentiation produces dark blue coloration of the organisms, which are recognizable as *H. pylori* on the basis of their characteristic morphology. Other stains such as cresyl fast violet[4], carbol fuchsin[5] and the more elaborate Gimenez stain[6] are equally capable of delineating the organisms.

At their most characteristic the organisms have a wavy, as opposed to spiral, configuration. The double wave produces an appearance which has been likened to a 'seagull in flight', but such is the pleomorphism exhibited by *H. pylori* that a wide variation in size and shape is observed. In particular, the coccoid form is impossible to distinguish from other organisms on purely morphological grounds. A sleeping seagull looks much like any other bird

from a distance! It should always be borne in mind that other spiral organisms may be present in gastric biopsies. Infection with *Gastrospirillum hominis* is an uncommon cause of chronic gastritis in humans[7]. These organisms are considerably longer than *H. pylori*, and have eight to ten tight spirals so that they are readily distinguishable from *H. pylori*, but can be differentiated from *Helicobacter felis* only by electron microscopy. The latter organism has been associated with chronic gastritis in a single case report[8].

The Gram stain has been used to identify *H. pylori* in smears of gastric biopsies or brushings of the mucosa and its modifications such as the Brown–Hopps[9], Brown–Brenn[10] and the half-Gram[11] are used by some laboratories for histological detection. The Brown–Hopps gives good colour distinction between the purple–red organisms and the surface mucus (pale straw colour) but the technique is more time-consuming and costly than the simplified histochemical stains. The half-Gram is a rapid method but there is little colour difference between the blue–black organisms and the violet tissue, although the same criticism can be levelled at the Giemsa stain.

Fluorescent staining using the non-specific nucleic acid-binding fluorochrome acridine orange on paraffin sections has been claimed to be easier to perform and quicker to examine than H&E or Giemsa[12]. Given that most histopathologists would not have immediate access to a fluorescence microscope it is unlikely that this approach will be widely employed. Furthermore the tissue, as well as the organisms, fluoresce with acridine orange, so that organisms in close contact with the epithelium are not readily discerned.

SPECIFIC TECHNIQUES

Specific fluorescence of *H. pylori* has been demonstrated in smears or frozen sections of gastric tissue in indirect immunofluorescence tests using monoclonal antibodies directed against the organism[13]. This approach has been generally superseded by immunohistochemical techniques. A polyclonal rabbit anti-*H. pylori* antibody has been used on paraffin sections in an indirect immunohistochemical method which, when compared with culture of parallel biopsies, yielded a positive predictive value of 84% and a negative value of 89%[14]. However, even better results are claimed for a commercially available monoclonal antibody raised against a flagellar epitope of *H. pylori* which can be used on paraffin sections of formalin-fixed material with a highly sensitive avidin–biotin immunoperoxidase labelling procedure[15]. Finally, *in situ* hybridization (ISH) has been successfully carried out in a few laboratories. Gavinet *et al.*[16] used sulphonated 1 kb fragments of sonicated *H. pylori* DNA as the probe to demonstrate the organisms *in vitro*, and van den Berg *et al.*[17] used biotinylated whole *H. pylori* DNA as probe on paraffin sections. However, the latter group studied sequential biopsies from only one patient, the specificity was not tested against other helicobacters, and some cross-reactivity with surface epithelial membranes was encountered. We have recently developed an *in situ* hybridization method[18] using as probe a biotinylated 109 base pair PCR product of *H. pylori*, the PCR primers

being directed at parts of the 16S rRNA fragment of the organism. Hybridization and detection of this probe yielded positive staining in all samples of *H. pylori*-associated gastritis tested, no signal with the closely related *Helicobacter felis* and *Gastrospirillum hominis* in tissue sections, and no cross-reactivity with gastric mucosa. ISH could be of value in the detection of very scanty organisms persisting after attempted eradication therapy, in the demonstration of coccoid forms, or for identifying organisms which have gained access to the lamina propria, where using conventional staining methods they are very difficult, if not impossible, to distinguish.

NEED FOR SPECIAL TECHNIQUES

Although a multiplicity of staining techniques is available for the detection of *H. pylori*, many pathologists question their value. It is frequently claimed that the organisms are readily identifiable in a routine H&E stain and there is no need for special stains. While heavy or moderate colonization by *H. pylori* is easily recognized in a good H&E preparation, scanty organisms may be missed. Madan *et al.*[10], in a comparison of staining techniques, found that only 83% of cases diagnosed as *H. pylori*-positive by Giemsa staining were deemed positive by H&E. Likewise, when 20 histopathologists examined a series of H&E sections for *H. pylori*[19], the overall detection rate was only 66% of those considered positive by a combination of Giemsa and Steiner staining. Furthermore, there was a substantial proportion of false-positive diagnoses based on H&E, resulting in a 10% overdiagnosis rate. The authors of the latter study concluded that the identification of *H. pylori* is unreliable if H&E alone is used.

COMPARISON OF STAINING METHODS

Having established that a special stain should be used for detection, which is the preferred method? There is little doubt that the silver impregnation techniques provide the clearest demonstration of the organisms; indeed the silver coating actually enlarges the bacteria, making them easier to see. Nevertheless, technical difficulties which result in background staining and precipitation give rise to interpretative problems and make this approach less than ideal. When the Warthin–Starry stain has been compared to simpler histochemical methods, a higher yield of positivity has been obtained with the latter. Potters *et al.*[20] found that 79% of culture positive cases stained positively with the Warthin–Starry stain while 94% were positive on modified Giemsa staining. Likewise in another study, only 81% of cases considered positive with the Wright–Giemsa stain were positive with the Warthin–Starry method[10]. However, whatever staining method is employed, histology should not be considered a 'gold standard' for the presence of *H. pylori.* Ultimately, culture and microbiological identification of the organism must stand as the final arbiter, but some centres seem unable to achieve the required standard of microbiological technique. Studies which have compared

histology with microbiology carried out using optimal conditions for collection, transportation and culture of specimens, have invariably shown a higher yield by culture[21]. Nevertheless, although one or more entire biopsies can be processed for culture, the technique is still open to sampling errors. It seems likely that well-validated breath tests, where the whole stomach lining can be 'sampled', will eventually emerge as the gold standard.

SAMPLING ERRORS

The value of histological detection of *H. pylori* would be severely compromised if colonization of the mucosa was patchy. Under these circumstances negative biopsies would be obtained from stomachs harbouring infection. Several studies have examined the sensitivity of histological detection. In a study where H&E staining was compared to culture[22], the results achieved by an expert observer were impressive — a sensitivity of 93%, a specificity of 87% and a positive predictive value of 84%. Wyatt[23] examined multiple antral and corpus biopsies (range 5–8) stained by the modified Giemsa stain from 50 unselected patients undergoing endoscopy. Of the 38 *H. pylori*-positive patients, 24 had the organisms in all biopsies, and 10 patients had organisms in all but one biopsy. Four patients had small numbers of *H. pylori* in one to three biopsies. The antrum was more evenly colonized than the corpus. Only 3.5% of antral biopsies from *H. pylori*-positive individuals gave a false-negative result, whereas 15.1% of corpus biopsies were false-negatives. In a similar study, Bayerdörffer *et al.*[24] examined 10 biopsies from standard sites in the antrum and corpus from 50 unselected patients and used the Warthin–Starry stain to detect *H. pylori.* They demonstrated a very close topographical relationship between colonization and active chronic gastritis. Thirty-two patients showed active chronic gastritis at one or more sites, and 227 of the 234 sites showing active gastritis (97%) were positive with the Warthin–Starry stain. It required up to four biopsies to establish positive *H. pylori* status in >95% of patients in their study, yet they conclude (in agreement with others) that in routine practice two antral biopsies are adequate to establish the presence of infection. Similarly, Morris *et al.*[25] showed that the examination of multiple sections from each biopsy would increase the yield of positive results, but such an approach is not practicable as a routine. This is in contrast to the requirements for diagnosis by culture, where it has been shown that only one biopsy is necessary to establish the correct *H. pylori* status with high probability[21].

Particular caution over sampling errors should be exercised in dealing with post-treatment biopsies where a proton-pump inhibitor has been used. Under such circumstances *H. pylori* may be cleared from the antrum but remain in small numbers deep within the corpus pits and the glands[26,27]. Thus, if histology is to be used to assess eradication it is mandatory to examine corpus biopsies.

INTER-OBSERVER AGREEMENT

There have been few formal studies on the reproducibility of a histological diagnosis of *H. pylori* infection. When four senior pathologists examined 82 antral biopsies stained by the Warthin–Starry method[28] they made a positive diagnosis in between 56% and 85% of the specimens, demonstrating significant heterogeneity. The pairwise kappa coefficients (a chance-corrected measure of agreement) ranged from 0.39 ('poor') to 0.82 ('excellent'). Specialists in this area may show less heterogeneity. When two experienced observers examined 280 individual biopsies from 50 patients stained by the Warthin–Starry technique[23] agreement reached 91% with a kappa value of 0.79 ('excellent'). Our recent experience has been even more satisfactory[29]. When three observers independently graded 64 sets of antral and corpus biopsies for *H. pylori* colonization as part of an evaluation of the Sydney System, there was only one disagreement over *H. pylori* status, giving a 99% probability of agreement on a diagnosis of *H. pylori*-associated chronic gastritis. Even overall agreement on the grade of *H. pylori* reached very satisfactory levels, namely 81% for the antrum (kappa = 0.739) and 84% for the corpus (kappa = 0.775). We therefore concluded that a high degree of reproducibility can be achieved.

CONCLUSION

Objectively, there is little to choose between many of the staining methods currently employed for the detection of *H. pylori.* The choice is a matter of personal judgement and laboratory practice, and no one technique can be considered 'best buy'. In addition to a special stain, the most valuable requirement is for a diligent, enthusiastic histopathologist who knows what to look for, and will spend several minutes scrutinizing a section before declaring it negative. Perhaps the performance of histopathologists would be improved by having a text hanging over their microscopes — 'Seek and ye shall find'[30].

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12
Microbiological tests

DAVID TOMPKINS

INTRODUCTION

Isolation by culture, simple microscopy and the biopsy urease tests are detection methods for *Helicobacter pylori* that have been refined over the past few years and are in common use, whereas the newer, molecular methods are research tools that may be widely adopted in the near future to aid diagnosis. The culture of *H. pylori* is often referred to as the 'gold standard' of methods for the detection of this microorganism because it is specific. However, the sensitivities of the methods used for the culture of gastric antral biopsies are, although greater than 95%, only equal to direct microscopic examination by histopathologists. For most bacterial infections culture is more sensitive than microscopy[1].

Molecular methods have been developed for the diagnosis of a variety of infectious diseases, including *H. pylori* infection[2]. DNA probes that will react directly with the nucleic acid of pathogenic microorganisms may be used on patient samples or following an initial culture procedure. The polymerase chain reaction (PCR) is used to multiply segments of nucleic acid specific for the infecting microorganisms to a level that is detectable either by a probe or by electrophoretic techniques. These methods should be more sensitive and rapid than culture.

The biopsy urease test has proved remarkably effective in practice for the detection of *H. pylori* in gastric biopsy specimens[3]. This is based on the early observation that *H. pylori* had substantial urease activity, greater than any other bacterial species previously described[4]. Ammonium ions generated from urea change a pH indicator. The test is crude but is simple, quick, inexpensive and relatively sensitive and specific.

The remainder of this chapter contains a review of the methods described above, and begins with a brief account of some of the characteristics of the organism to explain the basis of the practical procedures.

THE ORGANISM

Helicobacter pylori is a motile, Gram-negative bacillus which has a spiral morphology *in vivo*[2,5]. The normal habitat of this bacterium is the stomach of humans and other primates, where it survives closely attached to gastric epithelial cells beneath a layer of mucus[6]. In this niche the pH is virtually neutral and the bacteria are protected from the acidic gastric juice[7]. Many microorganisms survive in this site but, when present, *H. pylori* outnumbers the other species[8].

Microbial taxonomists have placed *H. pylori* in a genus with seven other helicobacters, and four of these also live in the stomachs of various mammals[9,10]. The production of substantial urease activity is a characteristic shared by the gastric-living helicobacters and may help them to survive in gastric acid[6,7]. A large, tightly spiralled organism (provisionally named *Gastrospirillum hominis*) is also found in the human stomach[11]. It is urease-positive and related to the helicobacters but has not yet been cultured *in vitro*[10,12].

The helicobacters are microaerophilic bacteria, and for their isolation *in vitro* a microaerobic atmosphere is required where the oxygen content is reduced to around 5%[6,7]. These conditions may reflect those found in the gastric mucosa[6]. The toxic effects of oxygen on *H. pylori* are not understood[7]. The function of blood or other substances in culture media, such as starch, charcoal or albumin, may be to absorb or inactivate toxic products or oxygen radicals[7]. Many bacteria grow best in an atmosphere which has an elevated concentration of carbon dioxide (5–10%). Although a microaerobic atmosphere gives better yields on primary isolation, *H. pylori* may be grown on subculture in humidified air with 6–10% CO_2 on media containing blood or other additives to improve aerotolerance[5,7].

Unlike those of most bacterial pathogens, *H. pylori* colonies are not visible before 2–3 days of incubation, and may take 7 or more days to appear after frozen storage or on primary isolation. However, as incubation proceeds, the spiral bacillary morphological types decrease in number and are replaced by non-culturable coccoid bodies[5,13]. The occurrence of coccoid forms in older cultures may be a response to ammonia production and increasing pH[13]. They may also occur on exposure to atmospheric oxygen[7]. *H. pylori* may survive in freshwater microcosms for up to a year in this coccoid state[7,9]. It also survives in water or saline in a culturable form for several days if the suspension is unaerated and chilled to less than 7°C, but becomes non-culturable within 24 h if held in these conditions at room temperature[7].

ENDOSCOPY ROOM TESTS

Some rapid tests for the presence of *H. pylori* in gastric biopsies are easily carried out in the endoscopy suite. The biopsy urease test was first described by McNulty and Wise[14], who showed that there was sufficient preformed urease activity in an *H. pylori*-infected biopsy specimen to produce a colour change in urea broth. Christensen's urea broth is used in microbiology

laboratories to detect urease activity in a wide variety of bacterial species. A large study using Christensen's broth showed it to have a specificity of 100% and sensitivity of 85% when compared with the detection of *H. pylori* by Gram stain, histology and culture[15]. The urease tests were read at up to 6 h and then at 24 h; 80% of the positives were detected in under 4 h. Many modifications of this original test have now been described, increasing the sensitivity and the speed of the reaction. All the tests have a specificity of about 100% and a sensitivity of over 70% at 1 h and of 90% at 24 h[3]. Some of the tests require incubation at 37°C or a higher temperature, which should improve their sensitivities[2,3]. Commercial test kits are available and are relatively expensive but highly accurate[3].

An 'ultra-rapid' test has been described which gives a result within 1 min and is inexpensive[16]. This test has a sensitivity of 89% and a specificity of 100% when compared with culture, out-performing the conventional urease test and Gram stain. The reagents have to be freshly prepared, or may be frozen in batches ready for use. Biopsies infected with *G. hominis* will commonly fail to give a positive urease test but may represent some of the 'false-positives' reported when *H. pylori* is not cultured[11,12,17]. Other contaminating, urease-positive bacteria have not been found to cause problems in practice[2,3].

A biopsy can be smeared on a glass slide and the material stained using Gram's method or a simple stain such as carbon fuchsin or acridine orange[2,5]. The characteristic S-shaped or spiral bacilli are observed microscopically. Sensitivities of over 90% have been claimed for the identification of *H. pylori* in Gram-stained smears if two or more biopsies are examined[18]. Obviously, microscopic examination is more technically demanding and time-consuming than a simple biopsy urease test. Immunofluorescence microscopy using monoclonal antibodies has been used to increase the specificity and sensitivity of microscopy[5,19]. However, this procedure is more suited to the laboratory than the endoscopy suite, and all fluorescence microscopy requires considerable technical expertise for accurate interpretation.

CULTURE OF GASTRIC MUCOSAL BIOPSIES

A variety of methods have been used to transport gastric biopsies, preserving *H. pylori* prior to culture. These include Stuart's transport medium, saline, 20% glucose solution and various broth media[2,5,7]. Broths have a theoretical advantage, as they will contain very little dissolved oxygen[7], and one comparative study showed better survival of *H. pylori* in nutrient broth than in saline or 20% glucose solution[20]. As stated above, the organism will survive for several days at 4–7°C so the transport medium should be chilled[7]. A biphasic transport system (a blood agar slope in a bottle with serum-enriched brucella broth), which may also be cultured, improved the isolation rate in one study but has not been evaluated by others[21]. When biopsies cannot be cultured immediately they may be refrigerated overnight or stored frozen at −70°C in broth with 20–25% glycerol as a cryopreservative, with minimal losses on storage for up to 6 months[2,5].

For optimum recovery of *H. pylori* the biopsy should be ground or finely minced in a little broth or saline using a tissue grinder or some other means such as two glass slides[5]. The homogenate is then used to inoculate solid agar media. Typical, spiral bacteria are not easily detected in Gram-stained films of this homogenate[18].

A variety of solid media have been used for the culture of *H. pylori*, usually incorporating 5–10% horse or sheep blood[7]. Selective antimicrobial agents may be added to inhibit the growth of contaminating bacteria and yeasts. Although serum, bovine serum albumin plus catalase, and starch have all been shown to be equivalent alternatives to blood for the continued subculture of *H. pylori*, they may not be as effective for primary isolation[7]. Furthermore the micriobiologist will usually want to use the media already available in the laboratory rather than making extra formulations. The agar plates should not be dried as *H. pylori* grows best on moist plates[2,5]. The plates may be stored for several days in an airtight container before use without detriment to the isolation rate[5].

A microaerobic atmosphere of 5% oxygen with 5–10% CO_2 is best for primary isolation. This gives better yields than humidified air with 5–10% CO_2, in a routine CO_2 incubator, which may be used for subcultures[2,5]. A high relative humidity is required for successful culture[2] and a suitable moist, microaerobic atmosphere can be generated in 'anaerobe' jars using commercial sachets or by evacuation of the jar and replacement of the air with a suitable gas mixture[7]. With the latter method, humidity may be increased by the inclusion of some blotting paper soaked with water in the jar. Like most human bacterial pathogens, *H. pylori* grows best at 37°C. Many strains will not grow at the higher temperature (42°C) used to selectively isolate *Campylobacter jejuni* and related organisms from faeces[2].

Two selective media are commonly used in Britain for the culture of thermophilic campylobacters. Skirrow's medium contains blood with trimethoprim, vancomycin and polymyxin to suppress the normal faecal flora. This medium has been used successfully for the isolation of *H. pylori*[22–27] although 5% of strains may be inhibited by the polymyxin[5]. The second is a charcoal medium (modified CCDA) which cannot be used to isolate *H. pylori* as it contains a cephalosporin and sodium desoxycholate, both of which inhibit its growth[7,28]. Selective media used for the isolation of *Neisseria gonorrhoeae* may double as selective media for *H. pylori*, although the mixture of antimicrobials will contain either polymyxin B or colistin, which will inhibit a small percentage of strains[7,18,27]. *H. pylori* is sensitive to clindamycin, so mixtures containing this agent should not be used[29].

The first selective antibiotic mixture specific for *H. pylori* included nalidixic acid (20 mg/l)[30], but as many as 14% of isolates may be inhibited by this agent[5]. A commercial antibiotic supplement is available (Unipath SR 147) which comprises vancomycin (10 mg/l), cefsulodin (5 mg/l), trimethoprim (5 mg/l) and amphotericin B (5 mg/l). This cocktail was originally used in a medium with 7% saponin-lysed horse blood in Columbia agar base[23]. The same antimicrobial agents have also been used in media containing either charcoal, horse serum and yeast extract or egg yolk emulsion in place of blood[27,31]. Mould growth can be troublesome in the moist conditions

Table 1 Comparative isolation rates (percentages) for *H. pylori* from gastric biopsies using different media

	Dent and McNulty[23]	*Tee et al.*[26]	*Glupczynski et al.*[27]
Chocolate agar	77	76	77
Skirrow's medium	94	96	NT
Thayer-Martin medium	NT	NT	86
Dent selective medium	100	89	NT
Brussels charcoal medium	NT	89	94

used for incubation, and may be controlled in media by the addition of cycloheximide (50 mg/l)[7]. Thorough cleaning of equipment is also required as moulds can be very difficult to eradicate.

The culture plates should be incubated for at least 7 days although colonies are usually visible at 4–5 days[2,5]. Colonies of *H. pylori* are usually 1–2 mm in diameter, glistening, translucent and produce slight haemolysis on blood agar media. On heated blood (chocolate) agar the colonies are tan-coloured[7]. Often the colonies have an irregular edge and are variable in size. From gastric biopsies the number of *H. pylori* colonies will usually vastly outnumber any contaminants, and oxidase, catalase and rapid urease tests (all positive) help to confirm the identity[2,5]. A pinpoint of colonial growth placed in a few drops of urea broth in the well of a microtitre tray will produce a colour change within a few seconds, and is diagnostic. Examination of a stained smear shows Gram-negative curved bacilli, with a few spiral and some U-shaped forms, and the bacteria are thicker than *C. jejuni*[5]. Round, coccoid forms predominate in older cultures[13].

The bacteria may die if exposed to atmospheric oxygen for more than an hour or so, and should be subcultured and returned to a microaerobic environment as quickly as possible[5,7]. Colonies are usually visible at 2–4 days on subculture, and for continued survival the subcultures should be carried out every 3–4 days[7].

The campylobacter supplement (FBP) cannot be used to improve aerotolerance of *H. pylori* as the sodium metabisulphite component is inhibitory to some strains at the concentration supplied (0.25 g/l)[30]. Vitox (Unipath) and Isovitalex (BBL) are chemically defined media supplements which may give more luxuriant and rapid growth of *H. pylori*[5,30]. Visualization of colonies may be improved by the incorporation in agar of triphenyl tetrazolium chloride (40 mg/l) as *H. pylori* colonies then develop a characteristic and unique golden sheen[32].

There are published comparisons of different media, but it is often difficult to evaluate the results. Slight modifications of ingredients may be made to the original formulations and terms like 'chocolate' agar may be misleading. However, results of three comparative studies are shown in Table 1.

A selective, enrichment technique has been described but it is impracticable for a busy diagnostic laboratory[33]. Growth of *H. pylori* in broth requires an adequate dispersion of a microaerobic or CO_2-enriched gas mixture throughout the culture medium[34,35]. This is achieved by using a small volume of broth in a large receptacle to increase the surface area exposed

Table 2 A culture method for biopsies

Transport	Brucella broth at 4°C
Homogenization	Grind/mince in transport broth
Media	Inoculate selective and non-selective media (fresh blood agar and Dent and McNulty selective agar[23] with 1% Vitox added)
Incubation	Microaerobic atmosphere at 37°C for 7 days
Identification	Gram stain, oxidase, catalase and rapid urease

to the atmosphere, or by mechanical agitation of the broth. Various basic broths have been used including brucella and brain–heart infusion broths[7]. Supplementation with fetal calf or horse serum is usual but serum may be replaced by starch or cyclodextrin[7,36]. There are no reports of practical, selective broth culture methods for *H. pylori* that could be used in diagnostic laboratories.

Conventional culture methods can have an accuracy of > 95% when carefully carried out by experienced workers[1,5]. In the author's laboratory a 96% agreement between culture and biopsy urease test results has been achieved using the methods outlined in Table 2.

CULTURE FROM OTHER SITES

Methods used for culture of *H. pylori* from gastric biopsies have been applied to other specimens. Gastric juice may be obtained via a nasogastric tube. Sensitivities of detection by juice culture vary widely, and are low when compared with histology and biopsy culture[8,37,38]. Dental plaque has been studied by a number of groups as a possible source of *H. pylori*, after the reported isolation from dental plaque of one of 71 endoscopy patients[39]. Although most groups have been unable to culture *H. pylori* from this site there has been one report of positive dental plaque cultures from each of 40 subjects, none of whom had gastrointestinal symptoms[40]. The isolation of *H. pylori* from faeces has also recently been described[41]. Bacteria were separated from the other faecal material and washed in buffer before routine culture on selective agar plates. *H. pylori* was cultured from the faeces of one healthy adult and nine of 23 randomly selected children in a Gambian village. It remains to be demonstrated whether this technique can be used to isolate *H. pylori* from the faeces of infected subjects in other parts of the world.

MOLECULAR METHODS

DNA probes specific for *H. pylori* DNA and 16SrRNA have been used to detect *H. pylori* in gastric biopsy tissues[42,43]. Although the probes are specific and sensitive (detecting 10^3–10^4 cells) the sensitivity is increased by the use of PCR. PCR tests for *H. pylori* include those based on a species-specific protein gene, the urease A gene, and 16SrRNA gene[44–48]. Sensitivities range from

100 to approximately two bacterial cells detected by these tests. Although the most sensitive test[48] also showed amplification of the related bacteria *H. mustelae* and *H. cinaedi* the other test based on the 16SrRNA gene, which detected as few as 10 bacterial cells, did not crossreact with these other helicobacters[45]. The PCR test has been used to detect *H. pylori* nucleic acid in gastric biopsy samples, gastric juice, dental plaque, saliva and faeces[44–51]. The test does not show whether the organisms are alive or dead. Problems that may arise with the PCR tests are contamination and false-negative results caused by inhibitory substances in tissues. As the tests are so sensitive, great care must be taken in the laboratory to avoid cross-contamination. It has also recently been shown that *H. pylori* DNA may be detected in fibreoptic endoscopes after cleaning and disinfection with glutaraldehyde[52].

FURTHER TESTING OF ISOLATED BACTERIA

If required for typing or further study, *H. pylori* strains may be stored frozen at −70°C in blood with or without 10% glycerol or in a variety of broths – again with 10–20% glycerol, which acts as a cryopreservative[5,7]. Typing of strains has proved to be difficult, and is reliant on molecular (DNA) methods[9]. Biochemical tests, other than the simple ones described above, are not required in diagnostic laboratories.

Culture of *H. pylori* is necessary for antibiotic sensitivity testing to be carried out. It has been shown that eradication rates with 'triple therapy' are compromised when organisms isolated pre-treatment are resistant to the metronidazole component[53,54]. Resistance to the macrolides and the tetracyclines also arises during exposure to these drugs[54,55]. Penicillin (amoxycillin) resistance has been reported so rarely as to make sensitivity testing to this agent unnecessary at present. Routine antibiotic sensitivities are tested most conveniently using the disc diffusion method, although this technique is better suited to rapidly growing bacteria. A heavy suspension of *H. pylori* is inoculated on a blood agar plate, discs applied, and the plate incubated at 37°C in a microaerobic atmosphere for 3 days. Very large zones of inhibition are seen with some of the antibiotics tested, and it is advisable to use no more than three discs in a standard Petri dish.

Resistance is most commonly seen with metronidazole. Most sensitive strains will produce a zone of inhibition of well over 20 mm diameter around a 5 μg disc[56]. Resistant mutant colonies can sometimes be observed within the zone of inhibition and will cause treatment failure. The break point (between strains classified as sensitive or resistant) for *H. pylori* to metronidazole is 8 mg/l, and for tests the antimicrobial agent can be incorporated into agar plates at this concentration[54,56]. The 'E-test' may be used to determine minimal inhibitory concentrations[29].

CHOICE OF TESTS

The correlation between the different methods for the diagnosis of gastric *H. pylori* infection is excellent[2]. Variations in the sensitivities of detection by

Table 3 Comparative sensitivities of biopsy urease tests, culture, and histological examination for the detection of *H. pylori* infection

Reference	*Biopsies*	*Sensitivity of method (%)*		
		Urease	*Culture*	*Histology*
McNulty and Wise[14]	1445	95	93	93
Morris *et al.*[24]	382	90	92	93
Glupczynski *et al.*[27]	203	77	94	81
Nichols *et al.*[57]	100	62	90	93
Logan *et al.*[58]	195	92	83	95
Schnell and Schubert[59]	160	92	70*	88
Rudensky *et al.*[60]	147	92	98	94

*Culture plates incubated for 4 days only

culture reported by different groups may reflect methods used, or the experience of the workers[5]. Comparative sensitivities of culture, histology and urease tests are shown in Table 3. Discrepancies between the techniques arise when there are few bacteria present in specimens[2]. There is a patchy distribution of *H. pylori* in the stomach[24,30]. A single biopsy has been taken for culture in most studies but, ideally, one from the body and one or two from the antrum should be examined[2,5]. One biopsy may be positive and a second negative in 14% of patients[24]. Other factors that interfere with the ability to culture *H. pylori* include the ingestion of topical anaesthetic or use of simethicone during endoscopy, recent use of antibiotics or H_2 antagonists and contamination of the biopsy forceps with disinfectant[5]. Eradication therapy may fail, and early sampling within 1 month of a course of treatment may then not detect the low numbers of bacteria remaining[1].

Culture of biopsies for *H. pylori* is relatively expensive, and is unnecessary unless antibiotic sensitivities are required. Gram strain examination is more demanding and less sensitive than biopsy urease testing[24,58]. This latter test, backed up by histology, is sufficient for the detection of *H. pylori* in most patients. Culture to detect live bacteria and the new ultrasensitive PCR tests will assist in the interpretation of therapeutic trials and epidemiological studies.

SUMMARY

Culture of gastric biopsies is an excellent method for the diagnosis of *H. pylori* infection if established techniques are applied with care. Viable bacteria are detected and antibiotic sensitivities may be tested. However, culture takes several days and the combination of rapid biopsy urease testing in the endoscopy suite with histology may be preferred if antibiotic sensitivities are not required. The place of the newer molecular methods for routine *H. pylori* detection has yet to be established, but PCR has already verified the presence of the organism in dental plaque and will be of value in establishing the mode of spread.

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DIAGNOSIS

13
Urea breath tests

DUNCAN BELL

INTRODUCTION

Detailed discussions of both the ^{13}C and ^{14}C urea breath tests have been published[1-3], including some fascinating historical perspectives. Our group has been using the ^{14}C urea breath test since 1986[4-17], but are relative newcomers to the ^{13}C test[18], which was originally described by Graham and colleagues in 1987[1,19].

The idea of developing a ^{14}C urea breath test to detect the presence or absence of *H. pylori* infection in patients' stomachs came as a direct result of two publications, both of which appeared in letter form in the *Lancet*. The first, from Tytgat's group, was the finding that *H. pylori* possessed an extremely powerful urease[20] and the second a report from Marshall and Langton that infected patients tended (because of the urease activity of the organism) to have lower urea and high ammonia concentration in their gastric juice than non-infected patients[21].

We[4], like others[1,22-24], realized that if differences in gastric urea/ammonia concentrations between infected and non-infected patients could be detected chemically[21,25] then it was likely that this phenomenon would be much more elegantly demonstrated using radiolabelled urea. We also predicted that radiolabelled urea would be considerably more sensitive than chemical measurement in the presence of a relatively light *H. pylori* infection where the total amount of gastric urease activity might be expected to be quite small.

When we initially applied to the Administration of Radioactive Substances Advisory Committee in 1986 for permission to use ^{14}C urea we had no idea how much of the isotope to use. We decided to initially suggest 0.4 MBq of ^{14}C urea, purely because this was the dose used for the original ^{14}C glycocholate breath test[26]. Our decision to take 10 min samples for 2 h after oral administration of the isotope was partially a reflection of our uncertainty regarding the likely pattern of $^{14}CO_2$ excretion in the patient's breath, and partly due to my previous experience as to the relative merits of single as opposed to multiple breath sampling gained while studying the use of ^{14}C aminopyrine as a test for both enzyme induction[27] and inhibition[28]. It is the

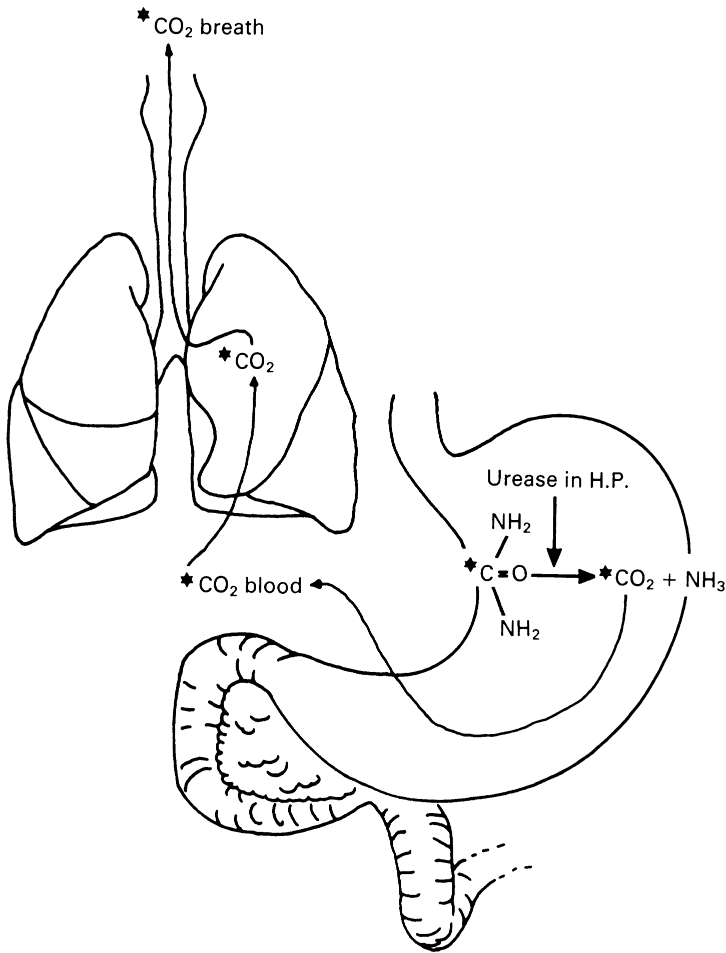

Fig. 1 Schematic drawing of the principle of the ^{13}C and ^{14}C urea breath test. When the labelled urea is administered orally, urea-derived labelled CO_2 appears in the breath of *H. pylori*-infected individuals. Reproduced by kind permission of author and publisher[2]

purpose of this chapter to discuss the developments and changes that have taken place in both the ^{13}C urea and ^{14}C urea breath tests since they were first described 6 years ago, as well as their relative merits and possible role in the overall management of the patient infected with *H. pylori*. The principle of the test, and an example of the result obtained using the ^{14}C urea breath test, are shown in Figs 1 and 2.

General principles that apply to all carbon breath tests

Klein and Graham[1] wrote that 'the principle of carbon breath tests is based on isotopic carbon tracers which exploit the concept of a target bond that

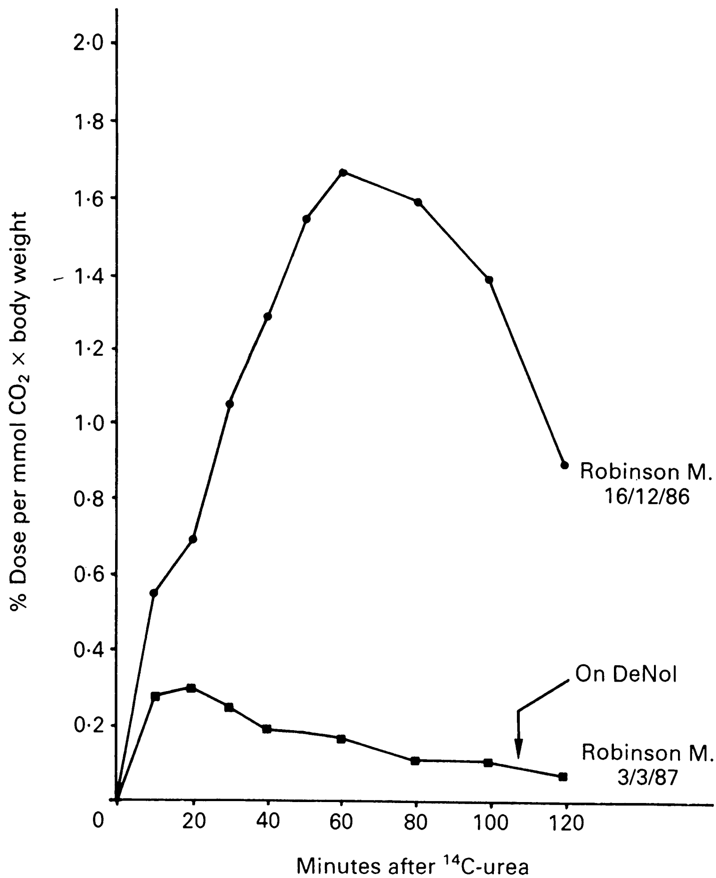

Fig. 2 Typical ^{14}C urea breath test result in an *H. pylori*-positive patient before, and again while taking the bismuth preparation De-Nol. Reproduced by kind permission of author and publisher[2]

links a low-molecular weight, isotopically labelled group to the remainder of the substrate molecule. Cleavage of the target bond releases the labelled moiety that may be, or may undergo subsequent conversion to, labelled carbon dioxide which is exhaled in the breath. The presence of the labelled carbon dioxide in breath signals the presence of an enzyme which acts on the target bond, and *the rate and extent of isotopic CO_2 production can be used to estimate the quantity of enzyme present*.'

COLLECTION AND MEASUREMENT OF $^{13}CO_2$ compared with $^{14}CO_2$

$^{13}CO_2$

The natural abundance of $^{14}CO_2$ is very low indeed, while that of $^{13}CO_2$ is relatively quite high. The ^{13}C is a natural non-radioactive isotope and can

be measured relative to ^{12}C by gas isotope ratio mass spectroscopy. For every million $^{12}CO_2$ molecules in breath, there are 11238 molecules of $^{13}CO_2$. This baseline $^{13}CO_2$ abundance can be measured with an analytical precision of 3 parts per million of total CO_2, or 0.03% of $^{13}CO_2$ concentration[1]. Because ^{13}C is expressed as a ratio to ^{12}C, the volume of expired CO_2 for any single sample is not critical, and the analysis can be done on < 0.1 ml of exhaled CO_2. As explained in greater detail elsewhere[3] the $^{13}C/^{12}C$ ratio is usually expressed as parts per thousand (per mil) relative to an international primary standard known as PDB calcium carbonate (PDB stands for ***Belemnitella americana*** and is extracted from the **P**e **D**ee formation of South Carolina (USA)). This curious standard was chosen because it has a ^{13}C to ^{12}C ratio close to the natural abundance of the two carbon isotopes. With PDB calcium carbonate the carbon isotope ratio is expressed as having a carbon isotope ratio of 0 per mil. Confusingly baseline breath samples are depleted in ^{13}C relative to PDB, and therefore frequently actually have negative per mil values.

During the day the baseline abundance of $^{13}CO_2$ normally fluctuates over a narrow range in response to dietary consumption of plant materials enriched in ^{13}C, such as corn and sugar cane. Stabilization of the baseline abundance is achieved by fasting the patient, or as in the case of the ^{13}C urea breath test, giving a test meal. Following ingestion of ^{13}C urea, patients infected by *H. pylori* frequently have a $^{13}C/^{12}C$ ratio in their breath that greatly exceeds that of PDB, thus giving a positive value. By expressing the results in terms of excess $^{13}CO_2$ excretion per mil by estimating the difference between before and after ^{13}C urea values it is possible to avoid this confusion. One of the advantages that both the ^{13}C and ^{14}C urea breath tests have over biopsy-based tests is that it is possible to define a cut-off between positive and negative results. One way of defining this is to take the ^{13}C urea breath tests of a large number of patients who have negative culture, rapid urease test, histology and serology, and calculate a normal range plus or minus 3 standard deviations from the mean and call this the upper limit of normal *H. pylori* negative range. In the study described by Logan[3] the cut-off achieved using this method was 4.9 per mil.

One of the major disadvantages of the use of ^{13}C breath tests in routine clinical practice was the problem of sample analysis. It was necessary to use isotope ratio mass spectrometers (IRMS) to measure ^{13}C enrichment in CO_2 because ^{13}C changes of less than 1 part per 1000 need to be determined. Before the actual ^{13}C measurement takes place, $^{13}CO_2$ must be purified from other breath gases. Previously, this had to be achieved by a cryogenic purification unit linked to the IRMS. Processing breath samples on such systems is slow (about 20 min per sample) and costly (0.5 l of liquid nitrogen required per sample). Furthermore, a specialist operator was necessary because, among other things, it requires a complex dual inlet IRMS for the final ^{13}C measurement.

One of the exciting developments in the field in the past few years has been the production of mass spectrometers specifically designed for ^{13}C gas analysis. One developed in the UK is a so-called automated breath ^{13}C breath analyser (ABCA) which utilizes fast and simple chromatographic

purification and a single-inlet IRMS. The ABCA consists of a Roboprep-G purification system, linked to a Tracermass stable isotope analyser (Europa Scientific, Crewe, UK). Briefly, each breath sample is automatically injected into the purification unit by a continuous flow of helium. Water vapour is removed by a magnesium perchlorate trap. A gas chromatograph (75°C) then separates CO_2 from N_2 and O_2 before the CO_2 is swept by the helium gas into the stable isotope analyser for measurement of ^{13}C enrichment. The breath samples are measured against a reference gas (5% CO_2, balance N_2) which has a delta value of −41.60 per 1000 when compared with PDB. As discussed above, the ^{13}C enrichment of the breath samples is then expressed as ^{13}C change (per 1000) over the patient's own baseline (0 min) delta ^{13}C value. It takes about 5 min to analyse a single sample and since gas chromatograph grade helium is considerably cheaper than liquid nitrogen the technique has a lower consumable cost, as well as a faster analytical time, compared with conventional IRMS.

With the latest 'state-of-the-art' ABCA-NT version of Europa Scientific's ^{13}C breath analyser the analytical cycle time has been further reduced to 2 min. When the ABCA-NT is linked to the appropriate autosampler it is possible to load 220 samples at a time. The samples themselves are stored in 13 ml septum-capped gas containers. The gas sample is then flushed from these containers by a stream of helium into the ABCA by a double-needle probe fitted to the autosampler.

When Graham and colleagues originally described their ^{13}C urea breath test in 1987 they opted to use 200 mg of ^{13}C urea[19]. As discussed more fully elsewhere[3], the 'European standard method' for the ^{13}C urea breath test employs just 100 mg of ^{13}C urea without loss of sensitivity or specificity. Our own group reported a study comparing the ^{13}C urea breath test with the ^{14}C urea breath test, and found that as little as 75 mg of ^{13}C urea could be used[18].

Most groups agree that giving a suitable test meal before administering the ^{13}C urea or ^{14}C urea is important in that it delays gastric emptying and prolongs the time that the bacterial urease is exposed to the labelled urea substrate. This becomes more critical if smaller doses of the labelled urea are employed and/or the patient has a light infection.

The ABCA is relatively inexpensive when compared with conventional MS but the price (May 1993) is still of the order of £70,000 plus VAT. Fortunately, however, one can now purchase ^{13}C urea breath test kits and then send off the breath samples to a commercial laboratory in the UK for analysis (see Appendix for address). The price of a single ^{13}C urea breath test including the cost of analysis works out at £28, but is somewhat less if a larger number of samples is being analysed. The 100 mg of ^{13}C urea is supplied in a gelatine capsule, which makes administration particularly easy.

$^{14}CO_2$ measurement

^{14}C, the radioactive isotope of carbon, is measured by liquid scintillation counting. To collect the breath sample at time 0 and at various time intervals

Table 1 Effective radiation dose a patient might receive from a 0.1 MBq ^{14}C urea breath test when compared with a number of other commonly performed isotopic and radiological examinations

Examination	*Effective dose* (μSv)	*Multiples of* ^{14}C *urea breath test*
^{14}C urea breath test (0.1 MBq)	8 (maximum)	1
^{14}C Glycocholate breath test (0.4 MBq)	100	12.5
Schilling test ^{57}Co and ^{58}Co	300	37.5
Chest X-ray	40	5.0
Skull X-ray	100	12.5
Thoracic spine X-ray	1080	135
Lumbar spine X-ray	2180	273
Plain abdominal X-ray	1400	175
IVU X-ray	4550	569
Barium meal X-ray	4630	579
Barium enema X-ray	8740	1093

after administration of the ^{14}C substrate the patient exhales through a tube of anhydrous calcium chloride into a vial containing 2 mmol of hyamine hydroxide (a trapping agent for CO_2) in 2 ml of ethanol with phenolphthalein as indicator; decolorization of the solution indicates the trapping of 2 mmol of exhaled CO_2. Then 10 ml of scintillant is added and the ^{14}C activity measured by liquid scintillation counting. A 1% standard is also counted and the activity expressed as the percentage of the administered dose per millimole of expired CO_2. This is multiplied by body weight (kg) to allow for endogenous CO_2 production. As has been reviewed elsewhere[2] several groups have argued that it is quite illogical to make allowance for endogenous CO_2 production by incorporating a 'fudge factor' involving the patient's weight. Indeed some groups no longer express their results as a percentage of administered dose/mmol CO_2 multiplied by weight in kg, preferring instead to use radioactive counts per minute (c.p.m.) since the correlation between the two is excellent.

The patient may need to exhale into the hyamine hydroxide for between 1 and 3 min to achieve decolorization of the solution (thus indicating the trapping of 2 mmol of exhaled CO_2). Marshall's group employ only 1 mmol of hyamine in their trapping fluid, which naturally halves the time the patient needs to blow into the solution since only 1 mmol of CO_2 is trapped.

When we started using the ^{14}C urea breath test we[4], like others[22], used 0.4 MBq of ^{14}C urea for our test. We soon came to realize[7] that the difference between the counts in the breath samples of our *H. pylori*-positive and negative patients was so great that we could reduce the amount of ^{14}C urea to only 0.2 MBq, and more recently have felt quite confident to reduce the administered radioactive dose still further to 0.1 MBq. This dose is similar to the 0.11 MBq dose used by the Amsterdam group[23]. We have calculated that if we employ 0.1 MBq of ^{14}C urea for our test, and use the data derived from the paper of Stubbs and Marshall[29] then the effective dose equivalent is between 4 μSv (micro Sieverts) (for a *H. pylori*-negative male) and 8 μSv for an *H. pylori*-positive female. In Table 1, for purposes of comparison, I have given the effective dose equivalent of a number of other radioactive

and radiological investigations. It can be seen that the maximum 8 μSv dose of radiation to which a patient's body is exposed is less than 1/500 of that which a patient receives when he/she has a barium meal and only 1/175 of the effective dose equivalent of a single plain abdominal X-ray. When it is recalled that the average person is exposed to some 1500 μSv per year in the form of natural radioactivity and cosmic radiation, one would need to have 187 of our 0.1 MBq ^{14}C urea breath tests in a year to double the patient's annual exposure to radioactivity.

I fully accept that in children and women of childbearing age it is preferable to use ^{13}C rather than ^{14}C urea breath tests, but for most situations the dose of radioactivity the patient receives when he/she has a ^{14}C urea breath test is so small as not to be a significant consideration. Work by Marshall's group[22] has indicated that the amount of urea metabolized to CO_2 depends on the presence or absence of HP in the gastric mucosal surface. Their data suggest that HP-negative subjects excrete 70% of the ingested urea intact in the urine with the remaining 30% of the ^{14}C being exhaled in the form of ^{14}C CO_2. In contrast, HP-positive patients excrete 40% of the ingested urea unchanged in the urine, with the remaining 60% being exhaled in the form of $^{14}CO_2$. Thus, when performing a ^{14}C urea breath test it is the urinary bladder wall that receives the highest dose of radioactivity. As discussed by Stubbs and Marshall[29] 'Prudent radiation practice seeks to reduce all absorbed doses to as low as reasonably achievable. Reducing the bladder voiding interval from 4.8 hours to 2 hours would decrease the dose to the urinary bladder wall by 42–53%'. In the light of this new data[29], we now encourage our patients to increase their fluid intake once the 40 min breath sample has been taken, and to try to empty their bladders within 2–3 h of receiving the ^{14}C urea.

If the clinician is working in a hospital that has a liquid scintillation counter, then the ^{14}C urea breath test would seem to have much to commend it. For rural GPs working in an area where the local hospital does not have a licence to perform the ^{14}C urea breath test, it would be preferable to purchase a small number of the commercially available ^{13}C urea breath test kits and have the samples sent through the post for analysis. In our hospital we have calculated that a ^{14}C urea breath test costs approximately £12 if one allows for technician time, time needed on scintillation counter, isotope, test meal, trapping agent, glassware, etc. Furthermore a beta-counter costs only a fraction of the £70,000-plus required to purchase a mass spectrometer, and can be used by the Medical Physics Department for other tests involving beta emitting isotopes.

Collection of breath samples — single sample or multiple?

A detailed discussion of the relative merits of multiple as opposed to single timed samples for both ^{13}C urea and ^{14}C urea breath tests has appeared with graphs[1–3]. Both tests have been modified for use with laboratory animals[30,31]. For most non-research purposes in humans it is quite acceptable to have a single baseline sample and one further sample at a single time

interval after administration of either the ^{13}C or ^{14}C-labelled urea.

In Ipswich we have opted to use the 40-min post-^{14}C urea breath sample, because in a large number of patients we found this gave the best correlation with the 2 h area under the curve (2 h AUC) value that we had originally employed by computing the AUC from samples taken at 10, 20, 30, 40, 50, 60, 80, 90 and 120 min. Rauws *et al.*[23] calculated the mean values of the ^{14}C activity for each time period between 10 min and 90 min after ingestion of the ^{14}C urea. A receiver operator characteristic (ROC) analysis was then performed by increasing stepwise the cut-off value of the test separating the *H. pylori*-positive from the *H. pylori*-negative subjects. Likelihood analysis revealed a most favourable cut-off level at 40 min. At that time, in the light of the Rauws paper, we reanalysed our own data and correlated the 2 h AUC with the single 40-min sample in 146 patients of known *H. pylori* status before any had received anti-helicobacter therapy. The correlation between the 2 h AUC and the much simpler 40-min test was 0.956 ($p < 0.01$)[7]. Our group now simply take the 40-min sample and compute the 2 h AUC. The cut-off we employ to separate *H. pylori*-positive from negative patients is a 2 h AUC of 40, which is equivalent to a 40-min value of 0.416% of administered dose/mmol CO_2 multiplied by body weight in kg. Using the single 40-min sample following ^{14}C urea, the Amsterdam group[23] separated their *H. pylori*-positive and negative patients with a sensitivity of 95% and specificity of 98% $^{14}CO_2$.

Similarly, with the ^{13}C urea breath test a baseline of breath sample 5 min after drinking a test meal of a mixture of 50 ml of Ensure and 50 ml of Calogen, followed by a second sample 30 min after drinking 100 mg of ^{13}C urea in 50 ml of water, gives results almost as good as more complex collection methods[3]. Earlier test systems relied on the patient exhaling into some form of inflatable bag from which the sample for analysis was drawn via a needle and syringe into a Vacutainer, which could then be posted off for analysis. With the single-sample technique for breath collection, many now simply use a disposable plastic straw, the tip of which is placed near the bottom of an opened 20 ml Vacutainer. When the patient gently exhales, air is displaced until condensation appears on the inside of the tube. The straw is removed immediately and the Vacutainer is resealed. In the commercially available kit available in the UK (see Appendix) the 100 mg of ^{13}C urea is conveniently contained in a gelatine capsule which is opened and the contents dropped into the 50 ml of drinking water. Sensitivity and specificity figures for the ^{13}C urea breath test are quoted as being of the order of 99% and 98% respectively[3].

TO ROLL OR NOT TO ROLL?

It is currently recommended that for both the ^{13}C and ^{14}C urea breath tests the patient lies down first on one side and then the other for a couple of minutes after ingestion of the labelled isotope, in an attempt to get the tracer dose evenly distributed over the lining of the stomach. I know of no good evidence that this actually makes any difference to sensitivity or specificity

of either test, and since it is particularly inconvenient for outpatients our own group in Ipswich stopped doing this several years ago, as indeed as Graham's group (personal communication).

APPLICATIONS OF BREATH TESTING

Epidemiological studies

Another chapter of this book is devoted to use of serology tests. My own view is that now much more specific and sensitive serology tests are available, this method is the cheapest and most effective way of screening large populations for epidemiological studies. Some fascinating studies have, however, been undertaken using ^{13}C urea breath tests, which may for instance be particularly useful in elderly patients in whom serology tests may be less reliable[1,3]. Urea breath tests do have the advantage over serology testing in reflecting current infection status, whereas it is known that serology tests for *H. pylori* may remain positive for several years in a significant percentage of patients whose infection has been eradicated.

Assessment of different anti-helicobacter eradication regimens

I believe that the greatest role for the non-invasive urea breath tests lies in the documentation as to whether or not a particular course of anti-helicobacter therapy has or has not successfully eradicated the patient's infection[1–3]. Using breath-testing techniques it rapidly became clear that many so-called successful eradications were in fact a reflection of temporary clearance[5], and many 'reinfections' following apparently successful eradication were examples of prolonged suppression of the organism followed by recrudescence and not true reinfection[16].

By definition *H. pylori* eradication implies waiting at least 1 month after stopping any candidate anti-helicobacter therapy before retesting. It is quite all right to retest the patient's helicobacter status while still on an H_2 antagonist drug, since this will not affect the test. In contrast, it is advised that omeprazole is stopped 1 month before trying to assess a patient's *H. pylori* status, since it itself can frequently dramatically suppress (but rarely eradicate) the infection[3,9].

SEROLOGY v UBT v ENDOSCOPIC BIOPSY — RAPID UREASE/CULTURE/HISTOLOGY

As stated above, serology would appear in most instances to be the best method of conducting epidemiological studies, while urea breath testing is to be preferred in the early (less than 1 year) follow-up of patients following attempted *H. pylori* eradication. If a patient is undergoing endoscopic examination clearly it is best to take samples at the same time. As discussed by Dixon's chapter on histological diagnosis, it can be difficult to detect the

organism in the presence of atrophic gastritis and intestinal metaplasia. Diagnosing the presence of *H. pylori* histologically tends to be most inaccurate when the infection is most scanty[32]. The urea breath test reflects *H. pylori* infection whatever part of the stomach is primarily affected, and, particularly in the presence of light infection of the type seen in the first few weeks after attempted *H. pylori* eradication, is in my view a better discriminator than histology. Culture and the rapid urease tests similarly may be falsely negative in the early post-antibiotic period. Culture is not routinely available, but is particularly useful if antibiotic sensitivity testing can also be incorporated. The rapid urease would seem to be particularly helpful in those endoscopy units where culture and/or histology is not available, and does of course offer a rapid 'bedside' test.

ANIMAL STUDIES

Animal models exist in both the ferret[30] and barrier-born pigs[31] that can be serially studied using breath test techniques. This should in future prove particularly useful in studying different anti-helicobacter drug regimens.

Acknowledgements

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Appendix: Commercial address for ^{13}C urea breath testing in UK

Bureau of Stable Isotopes Analysis, B.S.I.A. Ltd, 15 Brook Lane Business Centre, Brook Lane North, Brentford, Middx, TW8 0PP (Tel: 081-847 3955; Fax: 081-847 5053).

DIAGNOSIS

14 Serology

DIANE NEWELL and ALISON STACEY

INTRODUCTION

It is now well recognized that *Helicobacter pylori* has a significant role in the management of gastrointestinal disease. As a consequence, it is becoming increasingly important that clinicians have rapid access to accurate information about the *H. pylori* status of dyspeptic patients. Frequently this information will be needed prior to endoscopy, and therefore requires non-invasive diagnostic test systems. To date, two such non-invasive systems have been developed; serology and the breath test. So far serology has been the most useful technique for such assessment, though breath tests are now becoming more routinely available.

SERODIAGNOSIS WITH THE ENZYME-LINKED IMMUNOSORBENT ASSAY

Soon after the initial isolation of *H. pylori* it was demonstrated that the majority of infected patients elicit a significant circulating antibody response directed against the organism[1-3]. A number of techniques have been used to detect this serum antibody response, including bacterial agglutination, haemagglutination, immunofluorescence, latex agglutination, complement fixation tests and enzyme-linked immunosorbent assay (ELISA). The value of these serological tests in the assessment of *H. pylori* status is dependent on their accuracy, user-friendliness and cost. All these techniques are acceptable, but undoubtedly, on the basis of cost, simplicity and convenience, the ELISA is currently the method of choice.

The ELISA method involves the incubation of diluted human serum with *H. pylori* antigens immobilized onto the plastic of the wells of a multi-well plate. Unbound human antibodies are removed by washing. Any bound human antibodies are then detected by incubation with anti-human immunoglobulin antibodies conjugated to an enzyme, such as peroxidase or alkaline phosphatase. The enzyme indirectly bound to the plate is then visualized using a substrate which produces a coloured soluble product. The reaction

is stopped and the amount of colour in each well is measured using a multi-well plate reader.

Because the whole procedure can take as little as 2 h and the technique is suitable for semi-automation, hundreds of sera can be assayed per day. Moreover the increasing demand for readily accessible serological diagnosis has resulted in the recent development of a number of commercial kits from 'in-house' ELISAs. Thus the assay is no longer restricted to those more specialized laboratories which can produce antigen and standardize reagents.

Although the basic technology is the same in all the ELISAs developed, variations occur in a number of features including the antigen preparation used, the isotype of the antibodies detected and the method of quantitation and standardization.

ANTIGENS FOR SERODIAGNOSIS

A major contributor to the accuracy, in terms of sensitivity and specificity, of any ELISA is the properties of the antigen bound to the plastic plate. Initially, for research purposes, complex antigen preparations, such as sonicated whole organisms or acid-extracted surface proteins, were used[4]. Despite the crude nature of these antigenic preparations the circulating immune response was generally so substantial that *H. pylori*-positive and -negative patients could be differentiated in about 85–95% of cases[4]. However, the demand for better sensitivity and specificity has led to the identification and isolation of more suitable antigens[5–8].

These improved antigens are either partly or highly purified components of the organism. Some of the improved antigen preparations have now been adopted for routine use in so-called 'second-generation' serodiagnostic tests. The available methods of purification and the composition of such antigens have been reviewed elsewhere[5]. In principle, purification aims to enhance the proportion of materials antigenic during infection. To date the antigens considered the most useful candidates for such serodiagnostic tests include urease[9–11] and the 120 kDa protein[8,12], now known to be the cytotoxin-associated antigen. Purification also aims to reduce the contamination with antigens which have crossreactivity with other bacteria. A number of such crossreacting antigens are expressed by *H. pylori*, including heat-shock proteins and flagellin[13,14]. Even urease is not totally specific for *H. pylori*, as the related organism, *Gastrospirillum hominis*, also expresses an antigenically similar urease[15].

Unfortunately, antigen purification tends to result in a concomitant loss of sensitivity[9,16]. The reason for this is that not all patients produce antibodies directed against all the identified antigens of *H. pylori*. In fact, the spectrum of immune response during *H. pylori* infection is remarkably variable[17]. In order to guarantee the best sensitivity it is therefore necessary to ensure that a cocktail of specific antigens is present, rather than single purified antigens.

THE VALUE OF IMMUNOGLOBULIN CLASS ANALYSIS IN SERODIAGNOSIS

Some test systems detect IgA, as well as IgG, antibodies, and these can significantly increase sensitivity[18], though in some cases IgG antibodies are present in the absence of raised specific IgA[3]. It is possible that an assay which detects both classes may be the best method of enhancing the sensitivity. The value of IgG subclass detection has yet to be fully investigated; however, preliminary evidence suggests that specific circulating IgG_2 and IgG_4 are good predictors of infection.

THE QUANTITATION AND STANDARDIZATION OF ELISAs

The ELISA is quantitative, with the optical density of the colour product being directly proportional to the amount of antibody bound. There are a number of techniques available to quantitate ELISA results. Because the optical density is logarithmically related to antibody concentration, direct comparison of optical density is generally an incorrect reflection of the relative antibody levels in different sera. The use of a standard curve of optical density against antibody mass determined for the detection system used[3] is a more correct measure for comparison of sera. Serial dilution to give an endpoint titration is also acceptable, but is time-consuming and expensive. An alternative is the use of ELISA units, determined from a standard curve of optical density versus serially diluted standard human sera[6]. However, the dilution of sera may significantly alter the relative contribution of highly avid antibodies to the result. Moreover, the supply of the standard sera will always be limited.

SENSITIVITY AND SPECIFICITY: CUT-OFFS AND GREY ZONES

The sensitivity and specificity of any serological test is determined against patients of known disease status established by a 'gold standard'. For bacterial infections the gold standard should be culture, but in the case of *H. pylori* culture from biopsies is inherently difficult, due to both the fastidious nature of the organism and the patchy distribution of the colonization. Consequently, inadequate techniques for culture or sampling may result in anomalies. It is therefore strongly recommended that the 'gold standard' is, at least, a combination of culture and histology, on multiple biopsies. The addition of results from biopsy, rapid urease tests and breath tests will add to the accuracy of this 'gold standard'.

The threshold of seropositivity or 'cut-off' is unique to each test system used, and should therefore be determined for each test developed. Thresholds may be determined by established statistical methods using sera from uninfected individuals. However, this threshold may need to be adjusted, depending on the population under investigation[6]. This is because the immune spectrum and the antibody responses may vary significantly between certain groups. For example children in the developed world could be

expected to have a relatively immature immune response, and therefore produce only low levels of anti-*H. pylori* antibodies, especially during the early stages of infection. Fortunately these children have generally had little exposure to other infectious agents, so that the background is low. Therefore, for the best sensitivity the threshold for sera from children can be reduced[19,20]. Conversely, the background is generally increased in certain ethnic groups[21] or individuals in those geographic regions where heavy exposure to other microbial infections occurs. In these cases the threshold may need to be increased to optimize specificity.

Reproducibility and standardization are major problems in the comparison of serodiagnostic tests for *H. pylori*. Recently, surveys have been undertaken by the European Working Group on *H. pylori* Infections, using panels of sera from patients of established *H. pylori* status tested in a number of laboratories throughout Europe with both 'in-house' and commercial tests (R. Feldman, personal communication). The results (presented at the IV European Workshop on *H. pylori* Infections, Bologna, Italy, 1991 and the V European Workshop on *H. pylori* Infections, Dublin, Ireland, 1992) demonstrated that most tests, regardless of format or composition, had difficulties with defining sera from certain patients. It is generally accepted that about 5–10% of patients are in this problem category. Frequently these sera produce results close to, or at, the threshold of seropositivity, falling into the so-called 'grey zone' of some test systems; producing either inconclusive, false-positive or false-negative results.

False-negative or inconclusive results in infected patients are due to either:

1. Poor antibody responses because of immunocompromise. The elderly[22] and HIV-positive individuals may fall into this category.
2. Use of an inappropriate antigen. The antigen components may be too refined or produced from an antigenically incompatible strain, so that antibodies are produced but undetectable[6].
3. Acute infection prior to detectable circulating antibody production. Seroconversion may occur between 22 and 33 days after infection[23].

False-positive or inconclusive results in uninfected patients may be due to either:

1. The presence of circulating antibodies directed against other microorganisms which express antigens that crossreact with *H. pylori*, for example the flagella of *Campylobacter jejuni*.
2. Falling antibody levels following recovery from a previous *H. pylori* infection. There is no evidence of such a natural loss of infection occurring in symptomatic patients[24]. However, it has been suggested that there is some evidence for self-limiting disease in asymptomatic individuals[25], though this conclusion was probably inaccurate due to the use of an assay with a poor specificity. The frequency of self-limiting infection, though likely to be rare in adults, may be more common in children.

THE PERFORMANCE OF 'IN-HOUSE' ELISAs AND COMMERCIAL KITS

Interestingly, comparative studies indicate that the use of improved antigens has surprisingly little influence on the diagnostic value of the 'in-house' ELISAs of expert laboratories[26]. Nevertheless, over the past few years the sensitivity and specificity for the serodiagnosis of *H. pylori* infections have generally improved so that between 90% and 100% of patients are claimed to be accurately diagnosed by 'in-house' and commercially available tests[6]. However, it must be borne in mind that this accuracy has been established in groups of patients undergoing endoscopy. In such groups the high prevalence of infection relative to the normal population can significantly influence the performance indicators[6]. Additionally, the laboratories providing this information have substantial expertise in the selection of patients, execution of the tests and interpretation of the results. Thus the performance of such tests in the general population, and undertaken in endoscopy clinics or in the general practitioner surgery, may not necessarily reflect such accuracy.

In one of the recent studies by the European Working Group a comparison of 'in-house' ELISAs, from participating European laboratories, was made against several of the commercial kits available at that time (R. Feldman, personal communication). The results showed clearly that the kits could perform as well as in the 'in-house' ELISAs using the same set of sera. However, in the more recent and extensive survey, a major criterion of performance of the commercial kits tested was the competence of the laboratory (R. Feldman, personal communication). This indicates that although the technology is simple and the kits in a user-friendly format, the best performance may require laboratory expertise, thus limiting the usefulness of these test systems in a field environment.

APPLICATIONS OF SERODIAGNOSTIC ASSAYS

To date the major application of the ELISA has been seroepidemiology, but the serodiagnosis of dyspeptic patients and possibly treatment monitoring are potentially major uses of the test. To some extent the application determines the optimal test format and parameters, in particular the relative importance of sensitivity and specificity.

Seroepidemiological stidies

Because large populations are generally involved in epidemiological studies, the performance of the serological test is not crucial, though reproducibility and consistency are essential. In addition the requirement for statistical analysis means that epidemiologists prefer qualitative, rather than quantitative test results.

In epidemiological surveys specificity is considered a more critical parameter than sensitivity. Thus the presence of non-specific antibodies,

especially in those geographic regions where there is exposure to microbial infections with antigenically related organisms such as *Campylobacter jejuni*, are a major confounder. These antibodies may be eliminated by pre-absorption of the sera with whole *C. jejuni*.

Most importantly, as the ELISA performance varies with factors such as age and ethnic group, the performance of the test must be determined in the population under investigation. Alternatively age- and race-matched controls should always be included. Such requirements have led to the suggestion that tests specific for the chosen population, or even geographic region, may need to be developed in the future. Despite the inherent problems in studying widely varying populations, serology has been the major contributing tool to the descriptive epidemiology of the prevalence of infection in normal and symptomatic populations, as well as contributing to current understanding of the sources and routes of transmission. Moreover, serology is the only non-invasive technique which can provide the prospective as well as retrospective data needed for the investigation of the long-term consequences of infection.

Monitoring treatment

Serum antibody levels, of all isotypes, remain constant throughout infection[24] then decline relatively slowly following eradication of infection[27,28]. In order to monitor the efficacy of treatment it is therefore essential to analyse serial sera, preferably pre- as well as post-infection. A quantitative test system is essential because antibody levels may remain above the threshold even 12 months after successful treatment. In most patients 3 months post-treatment is the minimum time at which treatment success can be determined. Reinfection is accompanied by a rapid rise in titre[24]. It is therefore considered that serology is a reliable, albeit slow, indicator of effective treatment.

The antigen composition may have a significant influence on the ability of the serological test to detect falling antibody levels. For example, antibodies directed against the 120 kDa protein persist longer than antibodies against lower molecular weight antigens[29]. Thus the 120 kDa protein is not a recommended candidate for inclusion in ELISA antigen preparations for monitoring treatment.

As serial determinations are needed, the appropriate storage of the sera is important so that accurate comparisons of antibody levels can be made. Inappropriate storage and freeze–thaw cycles can lead to significant loss of antibody activity. Storage in small aliquots at −40°C, or in 50% glycerol at −20°C, should be adequate.

Serodiagnosis of dyspeptic patients

For diagnostic purposes in individual patients the performance of the serological test should, of course, be as close as possible to 100% sensitivity and specificity. This is undoubtedly impractical given the inherent variation

in host immune responses to infection. Nevertheless, using purified *H. pylori* proteins in a multi-component antigen preparation, and adjusting the threshold to take into account age and possible ethnic group, then performance approaching the optimum should be attainable. For diagnosis specificity and sensitivity are probably equally important, and constant monitoring of test performance is critical. For this reason any serodiagnostic test should be done in experienced laboratories by skilled staff. With the introduction of user-friendly, low-technology kits, marketed on the basis of saved time and cost, it seems likely that these will be increasingly used at the bedside or in the general practitioner's surgery. At this time caution must be counselled in the interpretation and use of these kits under such conditions.

Serology is increasingly useful in diagnosing *H. pylori*-related gastroduodenal disease in children[19,20]. However, as mentioned previously, the threshold for seropositivity may need to be adjusted from that already established for adults, in order to allow accurate detection of the low antibody levels expected in childhood acute infections.

There are two alternative strategies involved in the serodiagnosis of adult dyspeptic patients[30]; either the identification and exclusion of seronegative patients from further investigation by endoscopy or the selection and treatment of seropositive patients. With the increasing concern about the cost of endoscopies and patient load of endoscopy units in the United Kingdom such strategies have growing support.

Several retrospective and prospective studies have now investigated the outcome of a strategy of screening patients prior to endoscopy and deselecting seronegative patients from further investigation. It is assumed that seropositive individuals would include the vast majority of patients with peptic ulcer and gastric cancer. In order to minimize the risk of missing those patients with treatable gastric cancer, or those in potentially high-risk categories, it is recommended that only those individuals under a set age limit (40–45 years of age), with no previous clinical history of gastroduodenal disease and no prior treatment with NSAIDs, are screened.

A retrospective study[31] indicated that a significant proportion (about 42%) of younger (under 40 years), *H. pylori*-negative patients could be excluded from endoscopy. Such a strategy failed to detect only about 3% of all peptic ulcers. A more recent prospective study of 367 sequential patients[32] confirmed that if all seronegative patients under 45 years of age were excluded from endoscopy, then there would be a 17% reduction in endoscopic workload with no missed ulcers. Of course, such a strategy should be considered with caution, as opportunities to detect oesophageal disease may be missed.

The treatment of seropositive patients without prior endoscopy is still questionable. Improved drug regimens may encourage such a procedure, but the limited specificity of serodiagnosis, coupled with the widespread prevalence of asymptomatic infections, would indicate that confirmation of *H. pylori*-associated disease should be obtained prior to treatment.

SUMMARY AND CONCLUSION

With the recognition of the relationship between *H. pylori* infection and gastroduodenal disease, the development of non-invasive tests for diagnosis has provided useful tools for the investigation of dyspeptic patients. Without doubt serology is the most convenient approach. A number of serological methods have been developed, but the enzyme-linked immunosorbent assay (ELISA) has the advantage of simplicity, reliability and cost. Generally such tests have sensitivities and specificities of over 90%, and recent advances in the identification of appropriate antigens are now leading to significant improvements in these test criteria. Moreover, continued research will lead to more convenient test formats. With the availability of commercial kits it seems likely that serodiagnosis will become more widely used. The performance of these tests is largely dependent on the antigen used, but is also significantly influenced by patient age and ethnic group, and the laboratory competence and experience. Consideration must therefore be given to the role of such tests in the routine investigation of dyspeptic patients, both by general practitioners and within the gastroenterology clinic.

Serology already has a proven value in epidemiological studies, and is defining the role of this infection in gastroduodenal disease, especially gastric cancer. Quantitative ELISAs may also have some value in the longer-term monitoring of patient treatment. However, evidence is now accumulating which suggests that serology will be of most value in the pre-screening of selected groups (under 45 years, no clinical history and no NSAIDs) of seronegative dyspeptic patients, to allow a significant reduction (about 20%) in overall endoscopic workload. On the other hand the treatment of seropositive patients, without prior endoscopy, should be considered only with caution.

Serological tests are becoming increasingly useful tools in the non-invasive detection of *H. pylori*-associated disease. The sensitivity and specificity of the ELISA technique correlate well with the invasive methods of detection such as biopsy, bacterial culture and histology. In addition, the performance of the recently introduced commercial ELISA kits appears to compare favourably with current laboratory-based tests.

The antigens employed in the serodiagnosis of *H. pylori* have now been improved in terms of their sensitivity, specificity and reliability. Nevertheless, the development of better antigens is still necessary. Second-generation antigens are now being identified and adopted. Nevertheless, the unicomponent antigens, such as the purified 120 kDa antigen, whilst having good specificity, are always likely to have inadequate sensitivity in the ELISA. Future research efforts are likely to concentrate on the identification and isolation of multiple specific antigens from the organism, the establishment of techniques to prepare these antigens in reasonable quantities, for example by genetic engineering techniques, and the presentation of kits in simple user-friendly formats suitable for use in non-laboratory environments.

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15
Drug regimens

TONY AXON

INTRODUCTION

Helicobacter pylori is widely distributed throughout the general population and is responsible for considerable morbidity and mortality. In spite of the large number of subjects available for therapeutic trials, and the many investigators who have an interest in this area, no satisfactory medication has been identified which will eradicate the organism in all cases. Currently advocated regimens are in the main complicated and unwieldy, are associated with significant side-effects and are relatively expensive.

DIFFICULTIES WITH ERADICATION

The reasons for the difficulties experienced in identifying a suitable treatment reside in the nature of the organism, its ecological environment and the host response. The majority of infections with pathogenic organisms run a self-limiting course either to natural elimination of the parasite or to the death of the host. In most cases, therefore, when an antibiotic is given, total eradication of the organism by the antibiotic is not necessary since host factors work together with the antibiotic to eliminate the pathogen. Where *H. pylori* is concerned, however, the immunological response to the infection, though marked, is ineffective. Once the organism has successfully colonized the stomach it persists, and unless an antibiotic regimen is able to completely eradicate the organism, cessation of treatment is followed by recrudescence of infection to pretreatment levels within about a month. The pattern of infection is reminiscent of tuberculosis or subacute bacterial endocarditis where the organism inhabits a sanctuary zone difficult to access with antibiotics. Alternatively the organisms may have a relatively slow turnover rate, which renders them relatively resistant to antibiotic therapy. Unlike these two serious diseases, which untreated often lead to death, *H. pylori* is not sufficiently serious to warrant the long-term antibiotic therapy which these other conditions require.

The preferred site within the stomach for *H. pylori* is on the surface of the

epithelial cell and beneath the mucus layer. In this position it is protected from the acid milieu of the stomach by the underlying secretion of alkali. However, the mucus layer probably not only reduces the diffusion of acid into the epithelial cell, but also interferes with the diffusion of antibiotics. Similarly, as the organism is extracellular and outside the body proper, systemic antibiotics may also have difficulty in reaching it in its microenvironment. This does not apply to all antibiotics, and a recent study has shown that amoxycillin given intravenously eradicates *H. pylori* when given in combination with omeprazole[1]. These factors may play a role in preventing the organism from responding to antibiotics in the way that *in vitro* data would predict.

The secretion of acid causes the resting pH in the gastric lumen to be quite low. Many antibiotics are inactivated by a low pH, and this is thought by many to be the reason why certain antibiotics, particularly the penicillins, are relatively ineffective when given on their own.

The natural tendency of the stomach to empty means that, although very high levels of antibiotics within the lumen may be achieved intermittently, after the stomach has emptied concentrations may fall to below the level needed to eradicate an organism which has a relatively long reproduction cycle. Surprisingly little work has yet been done to study the intragastric and intramucosal concentrations of antibiotics, though one study indicated that high intramucosal concentrations were achieved for many[2]. The effect of preprandial or postprandial dosing, and the ideal vehicle in which the antibiotic should be given, are also subjects which have been under-researched.

PENICILLINS

Helicobacter pylori is sensitive to a wide variety of antibiotics *in vitro* (Table 1)[3], but when used on their own *in vivo* the results are uniformly disappointing. Although in some instances the organism may become resistant to the antibiotic, this does not appear to be the major reason for failure in most cases. The organism is, for example, very sensitive to penicillins *in vitro*, and does not appear to develop resistance to these drugs. However, although long-term treatment will suppress infection, recrudescence is usual when the medication is stopped[4]. It seems likely that where the penicillins are concerned, the main reason for their lack of efficacy is that they are less active at the low pH of the gastric lumen. This cannot, however, be the whole answer, because when given in combination with colloidal bismuth subcitrate and metronidazole, the addition of the amoxycillin increases the efficacy of the combination, and in particular seems to prevent the development of metronidazole resistance. Similarly, long-term phenoxymethylpenicillin, whilst not eradicating the organism, is sufficient to prevent the recurrence of duodenal ulcer during treatment[4].

Table 1 Susceptibilities of 50 isolates of *C. pyloridis* to 20 antimicrobial agents

Agent	*MIC (μg/ml)*[a]		
	Range	*50%*	*90%*
Penicillin G	0.015–0.12	0.06	0.12
Ampicillin	<0.003–0.03	0.015	0.03
Clavulanic acid	<0.01–0.64	0.16	0.64
Amoxicillin + clavulanic acid	<0.01–0.02	<0.01	0.01
Cephalothin	0.025–0.4	0.2	0.2
Cefotaxime	0.01–0.16	0.04	0.08
Cefsulodin	5.12–41	20.5	41
Streptomycin	0.04–1.28	0.32	0.64
Kanamycin	0.04–0.64	0.16	0.32
Tobramycin	0.04–0.64	0.08	0.16
Gentamicin	0.04–0.32	0.08	0.16
Erythromycin	0.1–0.8	0.2	0.4
Josamycin	0.4–1.6	0.8	0.8
Lincomycin	3.2–12.8	6.4	12.8
Chloramphenicol	2.0–8.0	2.0	4.0
Tetracycline	0.01–0.16	0.08	0.16
Rifampin	0.5–2.0	1.0	1.0
Pefloxacin	1.0–8.0	4.0	8.0
Colistin	2.0–6.4	8.0	32.0
Vancomycin	50.0–>100	>100	>100

[a]50% and 90%, MIC for 50% and 90% of the strains, respectively
From Lambert *et al.*, 1986[3]

NITROIMIDAZOLES

The group of antibiotics which have been most effective in *Helicobacter* eradication are the nitroimidazoles, tinidazole and metronidazole. These antibiotics are effective at an acid pH but unfortunately, when given on their own *in vivo*, resistance rapidly develops, and for this reason they should never be used alone. The high incidence of metronidazole resistance in *Helicobacter* infection in developing countries is almost certainly the result of the widespread use of these drugs for diarrhoeal disease. When combined with colloidal bismuth subcitrate, eradication rates of around 60% can usually be achieved[5]. Metronidazole has been used mainly in combination with colloidal bismuth subcitrate and a third antibiotic (usually amoxycillin or tetracycline hydrochloride), which gives eradication rates of around 90% (Table 2)[6].

Apart from the disadvantage of metronidazole resistance, there are other drawbacks to use of this antibiotic. Most studies have shown that moderately large doses of metronidazole have to be used for quite long periods to achieve good results, and this leads to side-effects. Most of the side-effects associated with the triple therapy regimen above are due to the metronidazole, which causes a metallic taste and nausea in many patients, and may be responsible for poor compliance.

BISMUTH

Colloidal bismuth subcitrate was the first antimicrobial compound noted to

be effective against *Helicobacter pylori in vivo*, and it was the observation that treatment of duodenal ulcer with this drug led to a reduced recurrence rate compared with other compounds which indirectly led to the discovery that *Helicobacter* eradication prevented ulcer relapse. Other bismuth compounds also have an anti-*Helicobacter* effect, but as with colloidal bismuth subcitrate their use as a monotherapy has been disappointing, eradicating in only 10–30% of individuals. Nevertheless, the bismuth compounds are an important component in triple therapy and help to prevent resistance developing to the nitroimidazoles much in the same way as para-aminosalicylic acid prevents isoniazid resistance in antitubercular combinations. In the doses used for *Helicobacter* eradication, there are no significant side-effects other than darkening of the stool, and the drug has been used for many years as a patent medicine without problems. Side-effects have occurred only when massive doses have been used for other purposes, such as ileostomy diarrhoea.

TRIPLE THERAPY

Triple therapy (Table 2)[6] has been the treatment of choice in recent years. A popular regimen has been colloidal bismuth subcitrate 120 mg four times a day, metronidazole 400 mg three times a day and tetracycline hydrochloride 500 mg four times a day. It is difficult to compare the results of different studies because they are not well controlled, and many of the regimens differ in dosage and length of treatment. However, in general tetracycline is preferred to amoxycillin as the third drug of the regimen because results appear to be slightly better, and penicillin allergy is not a problem. Unfortunately, to be effective the combination should be taken for at least 2 weeks, and this leads to significant side-effects in over 30% of individuals[7]. Common complaints are malaise, diarrhoea, nausea, a metallic taste and sore mouth, but pseudomembraneous colitis has also been reported. The complexity of the regimen and the problems with side-effects lead to a reduction in compliance. In one study lack of compliance was the major cause of failure[8]. It is very important, therefore, when prescribing triple therapy to make it clear to the patient why the drug is being taken, and to emphasize the importance of persisting with treatment and finishing the course, if necessary putting up with minor side-effects. A third problem, however, is metronidazole resistance, and when triple therapy fails, metronidazole resistance is usually found in the infecting organism. In Western countries the rates vary from about 10% to 20%, but in developing countries figures of up to 80% have been reported[9]. The presence of primary resistance does not necessarily mean that treatment will be ineffective. Between 30% and 60% of patients will still achieve eradication using the regimen in the presence of metronidazole resistance[7,10], but it is difficult to justify the use of this combination with its side-effects for such a disappointing eradication rate. As a result, some authorities have suggested that before prescribing triple therapy the organism should be cultured and sensitivities determined.

Table 2 Efficacy of 'triple therapy' in eradicating *H. pylori* from patients with either non-ulcer dyspepsia or duodenal ulceration

'Triple therapy'	*Duration (weeks)*	*Eradication* H. pylori (%)	*Reference*
TDB (1 tab) × 4 daily	2	90	75
Amoxycillin 500 mg × 3 daily	1		
Metronidazole 400 mg × 3 daily	1		
TDB (1 tab) × 4 daily	4	94	45
Metronidazole 200 mg × 4 daily	2		
Tetracycline 500 mg × 4 daily	4		
Amoxycillin 250 mg × 4 daily	4		
BSS (2 tabs) × 3 daily	2	90	76
Metronidazole 500 mg × 3 daily	2		
Amoxycillin 500 mg × 3 daily	2		
TDB (1 tab) × 4 daily	4	81	77
Amoxycillin 375 mg × 3 daily	4		
Metronidazole 500 mg × 3 daily	last 2 weeks		
TDB (1 tab) × 4 daily	4	65	78
Tetracycline 250 mg × 4 daily	4		
Metronidazole 200 mg × 4 daily	2		
TDB (1 tab) × 4 daily	4	74	64
Amoxycillin 500 mg × 3 daily	1		
Metronidazole 400 mg × 3 daily	1		
TDB (1 tab) × 4 daily	4	55	79
Amoxycillin 400 mg × 3 daily	1		
Metronidazole 400 mg × 3 daily	1		
TDB (1 tab) × 4 daily	1	71	80
Amoxycillin 375 mg × 3 daily	1		
Metronidazole 500 mg × 3 daily	1		
TDB (1 tab) × 4 daily	1	72	81
Amoxycillin 500 mg × 4 daily	1		
Metronidazole 400 mg × 5 daily	0.5		

TDB = Tripotassium dicitrato bismuthate; BSS = bismuth subsalicylate
From Heatley, 1992[6]

HELICOBACTER PYLORI AND GASTRIC ACID

It is the ability of *H. pylori* to cope with an acid environment which is largely responsible for its success as a parasite. The organism elaborates urease which splits the urea present in gastric juice, converting it to ammonium hydroxide, an alkaline solution which then surrounds the organism while it is present in the lumen of the stomach. It is subsequently able to gain access to the submucus layer which overlies the gastric epithelial cell, where the pH is close to neutral. Undoubtedly during its sojourn in the stomach, and particularly when moving from one area to another or making an exit to infect other hosts, it again has to run the gauntlet of very low pH. As a result of urease the organism is able to colonize an inhospitable environment and does not have to compete with other organisms which have not developed this ability to cope with acid. Indeed, its preferred niche under the mucus layer, where it is protected to a degree from macromolecules in the gastric lumen and is at the same time outside of the host tissues, may account for some of the problems experienced in finding antibiotic compounds that can

gain access to the microenvironment of the organism. It is not surprising that, when the acid milieu of the stomach is altered and hypochlorhydria supervenes, this should work to the disadvantage of the organism. It is well recognized that the organism is found less frequently and in smaller numbers in patients who have developed gastric atrophy with hypochlorhydria[11-15], and when the proton pump inhibitor omeprazole is exhibited the organism migrates from its preferred site in the gastric antrum to the more acidic area of the gastric corpus[16,17].

A number of mechanisms have been postulated to account for the observation that *Helicobacter* thrives less well in the hypochlorhydric stomach. Competition with other bacteria which are able to exist in the hypochlorhydric stomach is one possibility. Another is that immunoglobulins which are normally denatured by acid may be more effective against *Helicobacter* in a neutral environment[18]. Another possibility is that, with the reduction in gastric acid and the continued secretion of alkali by the gastric epithelial cell, the pH in the vicinity of the organism may rise. If at the same time the organism continues to elaborate urease and produce ammonia, it is possible that in the absence of neutralizing acid the organism may self-destruct as a result of ammonia toxicity[19,20]. In any event, it seems unlikely that hypochlorhydria itself will totally eradicate the organism because normal individuals may, during the course of their lives, go through periods when acid secretion is reduced, and under these circumstances *H. pylori* presumably has to accommodate to this. Although omeprazole therapy may reduce *Helicobacter* colonization, this therapy alone does not seem to eradicate it, and when the hypochlorhydric state is reversed the organism returns to its normal colonization pattern.

OMEPRAZOLE AND ANTIBIOTICS

The observation that omeprazole reduces *Helicobacter* colonization led to experiments with a combination of antibiotic therapy and omeprazole. Amoxycillin in particular has been widely used in attempts to eradicate the organism, and has met with variable success. The largest study, by Unge *et al.*[21], used omeprazole 40 mg daily and amoxycillin 750 mg twice daily for 14 days. In 157 patients the eradication rate was just over 50%. Better results, however, were achieved by Bayerdorffer *et al.*[22] in a small series of 27 patients. They used the larger dose of 40 mg twice daily and 2 g of amoxycillin twice daily for 10 days and achieved over 80% success. Two other studies using a twice-daily dose of omeprazole (20 mg rather than 40 mg) have achieved eradication rates of 90%[23,24], though the numbers in these studies were small. The most promising results therefore seem to be associated with a twice-daily dose of omeprazole which produces a better pH control over the 24 h. It also seems likely from the limited amount of data available that a 14-day regimen is more effective than 7 or 10 days. The regimen, therefore, that should produce reasonable results according to the German data would be 40 mg of omeprazole twice daily and amoxycillin 500 mg four times a day for 2 weeks.

The advantage of the omeprazole/amoxycillin combination over triple therapy is that there are fewer side-effects, the treatment regimen is simpler and metronidazole resistance is not a problem. The disadvantages are that fewer papers have been published on the subject, numbers are small and results over the range of published data are somewhat variable. There is also the problem of patients being allergic to penicillin. With these cautionary notes it seems that, if the data can be reproduced elsewhere, this form of therapy will prove to be the treatment of choice for *Helicobacter* eradication.

TREATMENT OF PROBLEM PATIENTS

The majority of patients will respond to either triple therapy (90%) or a combination of amoxycillin and high-dose omeprazole (?80%). When it has been decided to treat a patient with eradication therapy, one of these two combinations should be used in the first instance. If a patient is known to have a metronidazole-resistant organism, it is logical to start with the omeprazole/amoxycillin combination. If the patient fails to respond to either regimen, it is reasonable to switch to the other one for the second course if still indicated. When doing so, however, it is important to counsel the patients on the importance of taking the treatment as prescribed, because failure of compliance is probably the major reason for failure in most of the regimens. Patients usually conform if sufficient time is spent explaining the reasons why the different drugs are being used, and the problems associated with eradication. A small number of patients may fail to respond to both regimens, and under these circumstances other options should be considered. Hosking *et al.*[25] have shown that a 1-week course of quadruple therapy will eradicate infection in 95% of patients with duodenal ulcer. The standard triple therapy should therefore be given, and in addition omeprazole 40 mg twice daily. For those patients who are metronidazole-resistant and are allergic to penicillin, a further alternative is to use a combination of omeprazole and clarithromycin. Eradication rates have been as high as 80%[26], and a more recent small study obtained 100% eradication using clarithromycin 250 mg b.d., omeprazole 20 mg o.d. and tinidazole 500 mg b.d. for 1 week, though the incidence of nitromidazole resistance in this group was not recorded[27]. The problem with this combination is that secondary resistance may occur to clarithromycin in the event of failure, and it is probably desirable therefore to reserve this therapy for those who have failed with other treatment regimens.

SUMMARY

The ideal treatment regime for eradication of *Helicobacter* has not yet been identified. Acceptable results can be obtained by using triple therapy comprising colloidal bismuth subcitrate 120 mg four times a day, metronid-azole 400 mg three times a day and tetracycline hydrochloride 500 mg four times a day for 2 weeks. This regimen will eradicate *Helicobacter* in around

90% of individuals if they comply with the treatment. Unfortunately over 30% of individuals experience side-effects with this medication. An alternative form of therapy is a combination of omeprazole and amoxycillin. A suggested regimen is omeprazole 40 mg twice daily with amoxycillin 500 mg four times a day to be taken for 2 weeks. According to preliminary results this regimen should eradicate *H. pylori* from 80% of individuals. At present no other primary therapy should be used for *H. pylori* eradication because of the dangers of inducing resistance. In patients who have a metronidazole-resistant organism and are penicillin-sensitive, an alternative treatment is to use omeprazole together with clarithromycin, but this should be reserved for problem cases, because clarithromycin resistance will otherwise become widespread. Quadruple therapy combining omeprazole with standard triple therapy for a week has a very high eradication rate, but the complexity of the regime means that it should be relegated to a second-line treatment for patients who fail with the two primary treatments.

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TREATMENT

16 Vaccination possibilities and probabilities

MINHU CHEN and ADRIAN LEE

INTRODUCTION: THE NEED FOR IMMUNIZATION

Until recently it was considered that prophylactic measures against *Helicobacter pylori* were inappropriate as, although a high percentage of people in the world are infected with this organism, most are asymptomatic. In those in whom ulceration does occur, successful treatments are available, after which reinfection is unlikely. This situation has changed dramatically, however, with the growing realization that long-term infection with *H. pylori* is not without risk, i.e. if other factors are present inflammation may progress to gastric atrophy, a condition which has been shown to be a precursor lesion of gastric cancer[1–3]. Given that gastric cancer remains a major cause of death in many countries including China, Japan and Colombia, the development of an effective vaccine becomes desirable.

This chapter includes reflections on the attributes of any possible anti-*H. pylori* vaccine and also describes the results of early research on the use of immunization against *H. pylori*. These early studies suggest that immunization is an attainable goal, and have encouraged many groups throughout the world to become involved in vaccine development.

IS IMMUNIZATION AGAINST *H. PYLORI* LIKELY TO BE POSSIBLE?

There is a major difference between *H. pylori* infection and other microbial infections for which vaccines have been successfully developed[4,5]. The normal course of a bacterial infection is for the bacterium to become established and cause symptomatic disease of varying severity. Over time the patient dies or the infection resolves due to development of an immunity which eliminates the organism, or in the case of chronic disease such as tuberculosis causes sequestration. In contrast, unless infection is eradicated with antimicrobials, *H. pylori* once established in its ecological niche of the gastric mucus

remains in the stomach for the life of the host, continually inducing inflammation and occasionally causing symptoms. This lifelong infection occurs despite the mounting by the host of a vigorous immune response, i.e. *H. pylori* successfully inhabits the gastric mucosa in the presence of high levels of serum IgG and in the presence of large numbers of IgA-producing cells in the gastric mucosa[6–8].

Sceptics of the potential of immunization claim that immunization is unlikely to be effective against a bacterium that has not only successfully evolved to evade the immune response but may also possibly suppress certain components of this immunity[9]. It could be, however, that the immune response required to prevent infection is very different from that required to remove an already established infection. Indeed, there is precedent for this view. In a study of the immune response against the parasitic infection *Giardia duodenalis*, a small-bowel parasite of rodents, Mayrhofer and Sharma found that, despite the induction of an active immunity, *Giardia duodenalis* infected animals for life. However, when the infection was cleared by a course of metronidazole therapy, the animals became resistant to oral challenge of the parasite, while control previously uninfected rats were rapidly infected[10].

THE ATTRIBUTES OF THE IDEAL *H. PYLORI* VACCINE

If one takes into account the results of studies on the immune response against *H. pylori* and the experience gained from vaccine development against other intestinal pathogens it would appear that parenteral immunization against *H. pylori* would be unlikely to succeed, while an oral vaccine may be a more feasible approach.

H. pylori infects the gastric mucosa, and thus protection against this organism would necessarily require the induction of local immunity. Evidence in the literature supports this view. There are presently no parenteral vaccines available against any pathogen that induces effective mucosal immunity in the intestine. In addition, in the only published experiment on parenteral immunization against *H. pylori*, Eaton and Krakowka showed that not only did hyperimmunization via the parenteral route in gnotobiotic piglets induce no protective immunity, but that the pathology caused by *H. pylori* was actually exacerbated[11]. Another argument for induction of local immunity via the oral route is that IgA is essentially non-inflammatory[12]. Given that oral immunization is successful in preventing infection, other factors need to be considered. To be a successful vaccine the formulation would have to be able to stimulate the common mucosal immune system, possibly to a greater degree than that induced by natural infection. To do this would require an effective mucosal adjuvant. An adjuvant is an unrelated compound or carrier that facilitates the immune response against a particular antigen, resulting in a greater response than that obtained when the antigen is given alone. In addition, any oral vaccine would need to be inexpensive so that large numbers of persons in the developing world could be immunized.

CHOLERA TOXIN AS AN ADJUVANT

The first step in the long road to successful immunization against *H. pylori* came from some of the pioneering work of Elson, Holmgren and others who were involved in the development of a successful oral mucosal adjuvant[13,14]. To appreciate the significance of this work it is necessary to briefly review these early studies.

Until the early 1980s attempts to induce mucosal immunity with proteins administered by the oral route had been mainly unsuccessful. However, in early studies on cholera immunization it was found that oral immunization with cholera toxin, the toxic product produced by *Vibrio cholerae*, not only resulted in a specific secretory IgA response but oral tolerance against the *Vibrio* antigens was not induced[15,16]. When proteins which are highly immunogenic parenterally are given orally, a state of tolerance is induced, i.e. suppression of immunity against these antigens[16]. This is presumably a defence mechanism that protects our immune system from continued assault from ingested antigens. The mechanism by which cholera toxin somehow circumvents this normal process is as yet poorly understood[13]. The observation critical to the current theme is that co-administration of cholera toxin and other proteins appears to result in a secretory IgA response to the second antigen and abrogation of oral tolerance to that antigen. Thus the protein keyhole limpet haemocyanin (KLH), when given orally with cholera toxin, induced high levels of anti-KLH IgA, whereas on its own oral KLH was not immunogenic[17]. Nedrud *et al.* similarly showed that local antibody was produced if Sendai virus was given with cholera toxin[18,19]. What was of particular relevance and importance about the work of Nedrud *et al.* was that they actually demonstrated protection against challenge from viral infection following oral immunization with Sendai particles and cholera toxin adjuvant.

SUCCESSFUL ENHANCEMENT OF LOCAL IMMUNITY AGAINST *H. PYLORI*

A fine example of scientific serendipity saw the meeting of Nedrud with the paediatric gastroenterologist Steven Czinn. Czinn was one of the first to work on *H. pylori* in children, and had many interests in the epidemiology and pathogenesis of this organism[20–24]. A collaboration between these workers was set up and their first study was a repeat of Nedrud's Sendai experiments except that *H. pylori* was now used as antigen[25]. Whole-cell sonicates of *H. pylori* with or without cholera toxin were given intragastrically to mice or ferrets. Ferrets were included as they had previously been shown to harbour their own helicobacter, *Helicobacter mustelae*, and had been shown to be a useful model for *Helicobacter*-induced gastroduodenal disease[26,27]. The results of this study in mice are shown in Fig. 1. As can be seen, the inclusion of the cholera toxin induced a highly significant local IgA response. Results in the ferret were similar. From these results the authors concluded that it would be possible to develop an oral immunization protocol

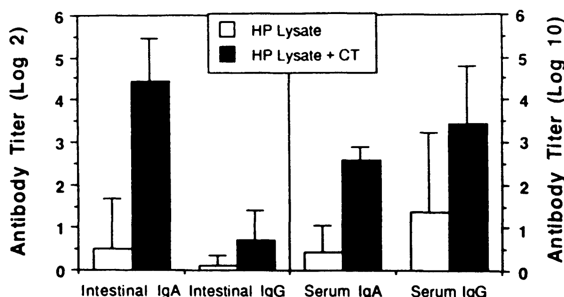

Figure 1 Murine antibody titres after oral immunization with *H. pylori*. Seronegative Balb/c mice (seven to 12 groups) were intragastrically immunized with four doses of 1 mg of *H. pylori* lysate either in the presence or in the absence of 10 μg of cholera toxin. Five days after the final immunization the animals were sacrificed and anti-*H. pylori* ELISA titres were determined for gastrointestinal secretions and sera. The error bars show standard deviations (copyright 1991 American Society for Microbiology)

for the prevention of *H. pylori* infection and associated gastritis. They were, however, unable to test the hypothesis that locally induced immunity would be protective, as *H. pylori* does not infect mice. Indeed, animal models of *H. pylori* infection are limited and are not practical for immunization studies. They include the gnotobiotic piglet, dog and primate[28,29]. Demonstration of the effectiveness of immunization against *Helicobacter* became possible only with the development of alternative models.

THE *HELICOBACTER FELIS* MOUSE MODEL

Just as in humans where *H. pylori* is the human adapted gastric helicobacter, other animals have their own evolved gastric bacteria[30]. Thus *H. mustelae* is the organism that colonizes the gastric mucosa of ferrets[31]. *Helicobacter felis* was first isolated from the gastric epithelium of a cat[32]. Subsequently, it was also grown from the canine gastric mucosa. Two human infections have been seen with this organism, both resulting in gastritis[33]. Rodents do not normally carry any *Helicobacter*-like organisms in their stomachs. However in 1989 Dick and colleagues showed that *H. felis* readily colonized the gastric mucosa of mice[34] (Fig. 2). As a result of this finding, the *H. felis* mouse model was developed and used as a screen for testing potential anti-*H. pylori* agents[35]. The relevance of this model was further reinforced when Lee *et al.* showed that infection of the germ-free mouse resulted in the induction of an active/chronic gastritis[36]. Subsequent studies have shown that long-term infection in mice follows the human progression from acute gastritis to active/chronic gastritis to chronic only gastritis with atrophy[37]. The validation of the *H. felis*-infected mouse as a good model of *H. pylori* infection provided an opportunity to test the Czinn–Nedrud hypothesis. Since that time, three groups have independently used the model to demonstrate that immunization is possible.

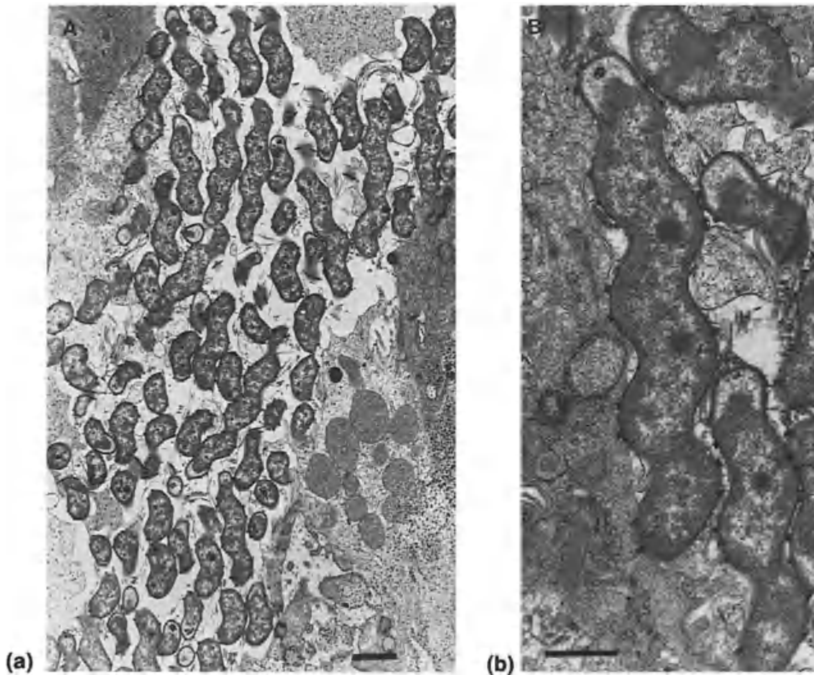

Figure 2 Gastric mucosa of a *Helicobacter felis*-infected mouse showing dense colonization of gastric pits with tightly spiralled *Helicobacter*. (**a**) Transmission electron micrograph (bar = 1 μm); (**b**) transmission electron micrograph (bar = 0.5 μm)

SUCCESSFUL IMMUNIZATION AGAINST GASTRIC *HELICOBACTER* INFECTION

The Chen–Lee experiments

The first published experiments of Chen and Lee repeated the Czinn–Nedrud protocol, except that an *H. felis* sonicate was used instead of *H. pylori* sonicate[38]. Specific pathogen-free mice were immunized with either *H. felis* sonicate alone, *H. felis* sonicate plus cholera toxin (CT) or saline. Three days after the completion of immunization, all animals were challenged with an oral dose of living *H. felis*. Eighteen of 19 animals in the group given the oral antigen plus cholera toxin were protected, whereas of those animals given the *H. felis* antigen alone or saline all were infected.

Based on these results a subsequent set of experiments was designed which included a cholera toxin alone control group and three groups of parenterally immunized animals (Table 1). The results of this study are shown in Table 2. This second study reaffirmed the findings of the first study, with 96% protection being achieved in animals that had been administered the *H. felis* sonicate plus CT. Hyperimmunization with intravenous *H. felis* gave no protection. However hyperimmunization by the intraperitoneal route resulted

Table 1 The experimental protocol of immunization against *H. felis* in SPF mice

Vaccine	*Immunization route*	*No. of animals*	*Immunization schedule*
Saline	Oral	21	Day 1, 3, 6, 30, 54
H. felis sonicate	Oral	21	Day 1, 3, 6, 30, 54
H. felis sonicate + CT	Oral	23	Day 1, 3, 6, 30, 54
CT alone	Oral	23	Day 1, 3, 6, 30, 54
H. felis whole cell	Intravenous	20	Week 1, 2, 4, 8, 12
H. felis whole cell	Intraperitoneal	22	Week 1, 2, 4, 8, 12

Table 2 Percentage protection and humoral and mucosal immunity levels obtained in the six different vaccination groups

Vaccine and immunization route	*Serum IgG*[a]	*Biliary IgA*[b]	*Protection* (%)
Saline (oral)	3.0 ± 0.3	3.3 ± 0.2	0
H. felis sonicate (oral)	3.2 ± 0.2	3.3 ± 0.3	0
H. felis sonicate + cholera toxin (oral)	3.4 ± 0.2*	4.1 ± 0.2*	96
Cholera toxin alone	3.1 ± 0.3	3.4 ± 0.2	9
H. felis whole cell (intravenous)	4.5 ± 0.2*	3.6 ± 0.2*	0
H. felis whole cell (intraperitoneal)	6.2 ± 0.3*	3.8 ± 0.3*	55

[a]Serum IgG ELISA units (log 10)
[b]Biliary IgA ELISA units (log 10)
*$p < 0.05$ (compared to saline group)

in 55% protection, a result which suggested that local immunity may play an important part in protection against infection. Indeed, when biliary IgA was measured in all these animals as an indicator of induction of mucosal immunity, increased biliary IgA levels were found to correlate with protection, a finding that again indicates the importance of mucosal immunity[39].

The Czinn–Nedrud experiments

Active protection

Czinn and Nedrud subsequently confirmed the finding of Chen and Lee, and in addition demonstrated significant titres of IgG and IgA antibodies in the serum, and IgA antibodies in the gastric lavage of immunized mice. This finding suggested that elevated IgA antibodies in the gastric secretions could result in protection against infection with *H. felis*. To determine if this was indeed the case Czinn and Nedrud carried out passive immunization studies using a specific IgA monoclonal antibody.

Passive immunization

In this study, mice were simultaneously administered (intragastrically) ascites containing the IgA monoclonal antibody 71-G_5-Ag (200 μl) along with 10^6 viable organisms of *H. felis*. Over the next 24 h a further three additional doses of monoclonal antibody were administered. A control group of animals was treated in a similar fashion except that a non-specific anti-Sendai virus

monoclonal or saline replaced the specific *H. felis* IgA monoclonal antibody. Of the seven experimental animals given the specific IgA monoclonal antibody, six were found to be protected and only one infected (14%), whereas of the 13 control animals receiving no antibody or irrelevant antibody, 70% were found to be infected. Although the numbers of animals used in this study were very small, the results are important.

When the specificity of this monoclonal antibody was investigated, it was found to react with the enzyme urease (Czinn, personal communication). Urease is known to be essential for colonization of the gastric mucosa by *H. pylori* and is considered an important virulence determinant[41].

The Michetti experiments

Important evidence that not only confirms the likely potential of immunization against *H. pylori* infection but also points the way to the most likely candiate for a vaccine to date comes from the work of Davin *et al.* This study demonstrated that significant protection against *H. felis* infection could be attained followed immunization with an oral dose of a recombinant urease subunit from *H. pylori* plus cholera toxin[42].

On the basis of the finding that oral immunization of mice with *H. felis* plus cholera toxin produced monoclonal IgA antibodies that recognized not only *H. felis* antigens but also many antigens of *H. pylori*, including the urease antigen of *H. pylori*, Davin *et al.* suggested that these bacteria shared antigenic determinants. This is consistent with the findings of Ferrero and Labigne at the Institute of Pasteur, who have shown an overall sequence homology of 80% between the ureases of *H. pylori* and *H. felis*[43].

In an attempt to determine if oral immunization of mice with *H. pylori* urease or a lysate of *H. pylori* elicited a protective mucosal immune response against *H. felis* infection in mice, Davin *et al.* orally immunized specific pathogen free Balb/c mice with either a purified *H. pylori* urease subunit coupled to hydroxyapatite plus cholera toxin or a sonicate of *H. pylori* plus cholera toxin at days 0, 7, 15 and 21. To obtain the urease antigen *Escherichia coli* was genetically manipulated such that it contained the UreB of *H. pylori* and synthesized Urease B subunit protein (Michetti, personal communication). The Ure B genes of *H. pylori* and *H. felis* have been shown to be 88% homologous. This protein was then coated onto 10 μm diameter spheres of hydroxyapatite. On days 28 and 30 the immunized mice, as well as a control group of unimmunized mice, were challenged with live *H. felis*. On day 35 the mice were sacrificed and antral biopsies removed for culture and a rapid biopsy urease test. Animals were considered to be infected when cultures and/or the urease test was positive.

Seven of ten animals immunized with urease plus cholera toxin were protected against infection with *H. felis*, a level of protection significantly higher (Fischer's exact test) than that found in animals immunized with the *H. pylori* sonicate plus CT 3/9 (33%) or in control animals 1/10 (10%).

As a result of this study the authors concluded that urease was a protective antigen of *H. pylori* and could therefore be used in an oral vaccine against

gastric infection by this organism. In addition, Davin *et al.* proposed that the *H. felis* mouse model would be suitable for the investigation of the immune response to *H. pylori*. This work, although to date only presented as an abstract, is extremely promising.

THE FUTURE OF IMMUNIZATION AGAINST *H. PYLORI*

Identification of the protective antigen

H. pylori-induced gastritis is an immunopathology, i.e. an inflammatory response induced by one or more *H. pylori* antigens[9]. One antigen that has been shown to be present deep in the gastric mucosa, and which has been suggested as participating in inflammation, is urease[44]. Because of this, it might be preferable to try and identify alternative protective antigens which are unlikely to have any inflammatory potential. Although there are plenty of other precedents of crude whole-cell vaccines in the literature, the crude sonicates used in the early studies described above are unlikely to be acceptable to the regulatory authorities. For these and other reasons there is a need to analyse successful protective responses more carefully, in order to identify protective antigens. Certainly the work of Davin *et al.*[42] would suggest that the urease subunit may be a possible candidate.

Although cholera toxin is the most effective oral adjuvant to date, its potential toxicity encourages research into other adjuvants. Possibilities include the Cholera B subunit[45], cholera toxoid[19] and non-cholera-based adjuvants[46–48].

Who to immunize

If one assumes from the encouraging experiments to date that immunization against *H. pylori* infection is a real possibility, then it is important to consider what groups would benefit the most from immunization.

Developing countries

The argument for *H. pylori* immunization in developing countries where gastric cancer remains a major killer is strong. In a most comprehensive analysis of gastric cancer in China, conducted in 1976, Chen *et al.* reported the gastric cancer mortality rate per 100 000 persons to be 22.19. Based on this figure, this means that on average 266,280 Chinese die per year of gastric cancer[49].

Large-scale epidemiological studies in China have shown that the first 3 years of life are critical in the acquisition of *H. pylori*. It has been postulated that this may be due to acquisition of the organism from the children's parents, an event that is increased by a high density of living[50]. After these early years the rate of acquisition of *H. pylori* is almost the same as in developed countries such as the USA or Australia, where acquisition rates of around 1% per year have been reported. It would appear therefore that

past childhood *H. pylori* is difficult to contract. Thus, if it were possible to immunize in early childhood, the prevalence of *H. pylori* infection should be drastically reduced. Even if there is a slow acquisition of *H. pylori* in later life, the number of people with long-term infection would be so low that one might expect to see up to 60% of gastric cancers being prevented.

Developed countries

The first reaction to a suggestion that vaccination against *H. pylori* should occur in a developed country is complete rejection. However, before one discards this proposal outright, one must give some consideration to the facts and the potential cost savings that such a step may incur. Some of the facts relating to gastroduodenal disease in developed countries are as follows.

1. The annual number of peptic ulcer disease patients in the USA = 2.5 million.
2. The annual number of visits to a US physician[51] for peptic ulcer disease = 5 million.
3. The estimated annual cost of peptic ulcer disease[51] in the USA = $3200 million.

To put these figures in perspective we may relate them to the number of deaths each year due to for example *Haemophilus influenzae*, a pathogen for which a vaccine has recently been introduced. In Australia each year, approximately 15 persons die of infection due to the bacterium *Haemophilus influenzae* compared to 700–1000 deaths due to peptic ulcer disease and gastric cancer, yet immunization against *H. influenzae* infection with the newly developed vaccine is being strongly promoted for all children in Australia[53–55]. Like *Haemophilus influenzae*, *H. pylori* is acquired in the first years of life. However, while children suffer serious disease with *H. influenzae* it is not until an older age that *H. pylori* tends to kill. Does this difference necessarily make the concept of vaccination against *H. pylori* infection in a developed country so unreasonable?

Parsonnet reflected on the value of antimicrobial intervention therapy for target risk groups such as *H. pylori*-positive Japanese living in Hawaii[56]. Could the argument not be made that such target groups would not benefit from *H. pylori* immunization?

CONCLUSION

The initial isolation of *Helicobacter pylori* from gastric biopsies of patients with upper gastrointestinal symptoms, and the insistence 10 years ago by Robin Warren and Barry Marshall[57,58] that this organism played a role in gastroduodenal disease has revolutionized gastroenterology. As Graham (personal communication) has observed, the time has come to simply view *H. pylori*-associated peptic ulcer disease as another infectious disease that demands treatment as does tuberculosis. The implication that this same organism plays a role in gastric cancer also demands that we search for methods of prophylaxis rather than simply relying on cure. The first steps

towards a prophylactic vaccine have been made. The next 10 years will determine if the possible becomes the probable and then the actual. Immunization against *H. pylori* infection is an attainable goal which has the potential to eliminate two of the major gastroduodenal diseases seen today.

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CONCLUSION

17 Conceptual evolution from gastritis, to peptic ulcer and gastric cancer, to 'cystitis of the stomach' and, finally, to a smallpox equivalent

DAVID GRAHAM

INTRODUCTION

Infection with the bacterium *Helicobacter pylori* is now recognized as the most common cause of gastritis. Several decades ago it was recognized that there was a strong correlation between atrophic gastritis and gastric carcinoma, and gastritis became an important subject of scientific inquiry. Gastric carcinoma continues to evoke great interest because it remains one of the major causes of cancer death worldwide.

Studies of the epidemiology of gastritis showed an association with low socioeconomic class, age, peptic ulcer disease, gastric carcinoma, and pernicious anaemia. Studies of the relationship between gastritis and gastric carcinoma demonstrated that environmental factor(s) were involved in development of gastritis and in progression from atrophic gastritis to gastric cancer. Despite a large number of studies that implicated environmental factors and provided clues, such as differences in prevalence of gastritis depending on socioeconomic class and ethnic group, no common theme or factor emerged. The suggestion that an infection with a bacterium could be the major cause of gastritis prompted little interest until the organism was grown in culture. Evidence that ingestion of *H. pylori* caused gastritis, and that the gastritis regressed or even healed following therapy, stimulated great interest, and has resulted in a virtual flood of new investigations. We now know that the epidemiology of gastritis was a surrogate for the epidemiology of *H. pylori* infection[1] (Fig. 1).

The initial focus of *H. pylori*-related research was to develop methods to detect the infection non-invasively, and to study its epidemiology. The next focus was to identify methods to eradicate *H. pylori* infection reliably. Progress in development of detection methods was rapid and investigators

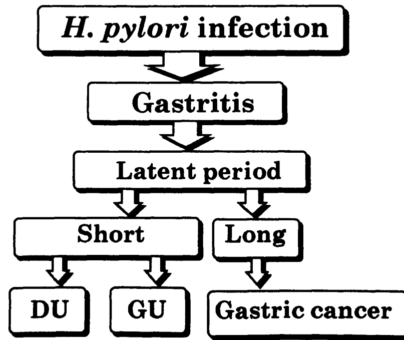

Fig. 1 *H. pylori* infection causes gastritis which may remain asymptomatic. Approximately 10–20% of those who become infected develop peptic ulcer disease. A much smaller proportion develop gastric cancer. A long duration of infection and other, as yet unidentified, co-factors are required for this expression of the disease

were soon provided the tools necessary to identify the presence of *H. pylori* infection[2]. In contrast to these successes, the early treatment studies resulted in low rates of eradication[3]. Development of multi-drug combinations, such as the triple therapies first described by Tom Borody, finally provided the tools needed to determine the effects of eradication of *H. pylori* infection[3,4].

The general approach to investigation of the role of *H. pylori* in a disease has been to eradicate *H. pylori* infection and then ascertain whether the natural history of the disease has been altered. At least a score of studies of *H. pylori* eradication in duodenal ulcer have been performed and, despite the differences in protocols, the results have been consistent; eradication of *H. pylori* cures duodenal ulcer disease[5–18]. Although details of ulcer pathogenesis remain unclear, the studies have consistently shown that eradication of *H. pylori* infection leads to acceleration of ulcer healing, prevention of relapse, prevention of ulcer complications, and obviates the need for maintenance antisecretory therapy. All but the most strident critics have been convinced that the majority of peptic ulcers are caused by *H. pylori* infection.

Evidence that *H. pylori* played a major role in peptic ulcer disease changed the questions from how to treat to whether to treat and whom to treat. Recommendations for therapy for use on anti-*H. pylori* have changed rapidly. Authorities initially proposed that the decision to treat should be based on the severity and duration of ulcer disease. For example, because effective and safe antisecretory therapy is available, it was recommended that antimicrobial therapy be reserved only for those with complicated ulcer disease, those with frequent recurrence, and those who might otherwise be surgical candidates.

We are now in the midst of another change in our perception of *H. pylori* infection; one in which *H. pylori* infection as one factor in the pathogenesis of peptic ulcer is being replaced by the realization that peptic ulcer disease is simply one of many different manifestations of an infectious disease (Fig. 2). This new appreciation has tremendous implications. First, we recognize

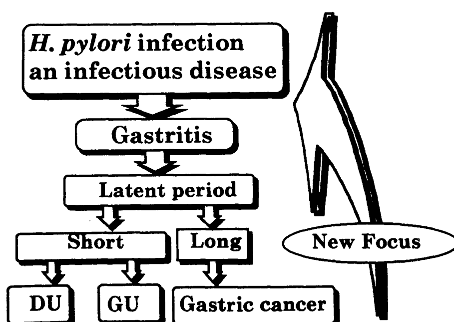

Fig. 2 The focus is moving away from single manifestations of *H. pylori* infection towards viewing *H. pylori* within the broad category of infectious disease

Table 1 Analogy of the factors important for making the decision to use antimicrobial therapy for a patient with an *H. pylori* duodenal ulcer and a patient with a urinary tract infection

Parameter	*Urinary tract infection*	*Duodenal ulcer*
Severity important	No	No
Duration important	No	No
Symptomatic therapy available?	Pyridium	Antacids
Suppressive therapy available?	Mandelamine	H_2-antagonists
Effective antimicrobial therapy available?	Yes	Yes
Cure guaranteed?	No	No
Recurrence possible?	Yes	Yes
Pathogenesis understood?	No	No

there is no need to invent new ways of considering or treating bacterial infections. Conceptually, *H. pylori* gastritis is not different from *E. coli* urinary tract infections. Just as a urinary tract infection can present with varied manifestations, such as asymptomatic bacilluria, papillary necrosis, or renal abscess, *H. pylori* infection also can present in different guises. The urinary tract analogy is useful to help decide what to do for a particular patient (Table 1). Although the example is an oversimplification, *H. pylori* infection can be considered to be analogous to cystitis, i.e. 'cystitis of the stomach'.

Once we accept that *H. pylori* infection is an infectious disease with a number of different manifestations (i.e. gastritis, peptic ulcer disease, and one form of gastric cancer), it is possible to move beyond focusing on a single manifestation such as peptic ulcer disease towards viewing *H. pylori* within the broad category of infectious disease. It is a short leap to the conclusion that *H. pylori* infection is really a public health problem, and that the proper questions are its effect on our population and whether it be eliminated (Fig. 3).

Just as the analogy of *H. pylori* gastritis and *E. coli* cystitis is useful to help understand the approach to a particular patient, an analogy of *H. pylori* to smallpox virus illustrates the potential for eradication of *H. pylori* from

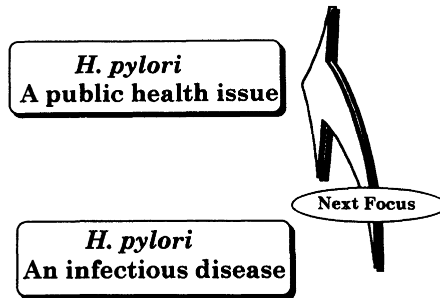

Fig. 3 The approach to *H. pylori* infection will soon be based on the realization that, despite the dramatic nature of some of its manifestations, *H. pylori* infection is really a public health problem. The pertinent questions will then be what are its effects on our population and how to eradicate *H. pylori* from mankind

mankind. The host range for *H. pylori* is limited to humans, with no known animal reservoirs. Higher standards of living and improved sanitation result in a reduction in transmission and a fall in prevalence of *H. pylori* infection and of *H. pylori*-associated diseases[1,19,20]. Although the route(s) of transmission from host to host are unclear, and we have not yet identified whether there are particularly weak links in the transmission chain, it should be possible to eradicate *H. pylori* infection from populations of developed countries. Inexpensive methods to detect *H. pylori* are available, but the best strategy to eliminate *H. pylori* infection has not yet been developed. One possibility is case detection and administration of antimicrobial therapy with or without adjuvants, such as exogenously administered antibodies. There is considerable interest in the potential use of vaccines to prevent or reduce transmission[21–23]. However, vaccine development has not progressed sufficiently to predict whether vaccination will play a significant role in *H. pylori* eradication programmes.

Consideration of such eradication programmes is especially germane to countries that have recently become industrialized, such as Japan, Saudi Arabia, and Korea. These countries have 'first world economies' and 'third world stomachs', as evidenced by gastric cancer and peptic ulcer being major causes of morbidity, mortality, and health care expenditures. It is evident that the pattern of prevalence of infection in these countries will change over time, with each successive generation experiencing a progressively lower rate of acquisition of *H. pylori* infection. It should not be necessary to wait several generations for the natural and gradual loss of *H. pylori* infection from these societies. It should be possible to turn the clock ahead and experience the future now. *H. pylori* gastritis is caused by a bacterial infection, and that infection can be eradicated.

Until recently it would have been unthinkable to suggest that it might be possible to eliminate the scourge of peptic ulcers and gastric carcinoma. This possibility now deserves serious consideration. The tools needed to begin an eradication programme are available, but it is important to address how to do so in a manner that is cost-effective. These are the challenges that lie ahead.

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