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Helicobacter pylori New diagnostic tools. Clinical and experimental studies on local and systemic immune response

Minireview based on a doctoral thesis

Lars Engstrand

Department of Clinical Microbiology, University Hospital, Uppsala, Sweden

INTRODUCTION

Helicobacter pylori - a historical review

The presence of gastric spiral bacteria in the stomach of animals was described before the turn of the century (4, 132). In humans such bacteria were first reported in 1906 by Kreinitz (87), who isolated "spirochaetes" from the stomach of a patient with gastric carcinoma. Thirty years later, using a silver staining method, Doenges described "gastric spirochaetes" in tissue specimens from human stomachs (24). These findings were retrospectively associated with gastritis. In 1940 Freedberg and Barron (51) found spiral bacteria in the gastric mucosa from resected stomachs. These organisms were found to be associated with ulceration, but the authors concluded that the bacteria colonized the mucosa as non-pathogenic opportunists. Palmer (118) examined over 1000 human gastric biopsy specimens but did not use silver stain. He was unable to demonstrate spiral organisms and decided that the organisms reported above must, after all, be *post mortem* contaminants. Since that time, gastric spiral bacteria have rarely been mentioned in the literature.

Much research work has focused on the significance of the urease activity on the surface of the gastric mucosa and its association with peptic ulcer disease. Fitzgerald and Murphy (47) concluded that the gastric mucosa produced urease as a defence against acid. However, the material studied was resected stomachs obtained after gastric surgery. Because most gastric surgery was performed on patients with peptic ulcer disease, it is most likely that these specimens contained *Helicobacter pylori* (*H. pylori*). Neither the recognition that the urease activity disappeared from the stomach when patients were treated with tetracycline (92) nor the absence of gastric urease in germ-free animals (21) awoke the idea that gastric urease might be of bacterial origin. Here again, although the origin and significance of gastric urease remained unknown, interest in gastric urease waned.

When large-scale sampling of fresh material was made possible by the introduction of flexible fiberoptic endoscopy techniques in the 1970s, Steer and Colin-Jones (139) reported presence of Gram-negative bacilli in 80% of their patients with gastric ulcer - but not on normal mucosa. Attempts to culture these organisms resulted in growth of *Pseudomonas aeruginosa* which was mistakenly identified as the gastric spiral bacterium. Isolation of *Campylobacter jejuni* and related organisms under micro-aerophilic conditions was unknown in most clinical laboratories at that time (20, 135). Thus, *H. pylori* did not grow and was not detected.

Robin Warren, a pathologist at Royal Perth Hospital, Western Australia, observed in 1979 gastric spiral bacteria on histologic sections from patients with gastritis. He introduced Barry Marshall, a trainee in internal medicine, and asked him to further investigate the bacteria he had observed. They became certain that the bacterium was clinically relevant and in April 1982, after the four-day Easter holiday, the spiral organism was cultured for the first time using standard campylobacter media. This was of importance, since attempts to culture the bacteria had been unsuccessful for 6 months due to the laboratory routine where the plates were discarded after 48 h if no growth was visible (Marshall 1989, personal communication). They published their data in 1983 and 1984 (99, 146).

One problem was that the organisms did not resemble any known bacterial species. The organisms were named campylobacter-like organisms (CLO) because of their morphological similarity to members of this genus. The name *Campylobacter pyloridis* was proposed (98) and amended to *Campylobacter pylori* (97). According to specific taxonomic criteria the organism was later recognized as a new genus and therefore given a new name: *Helicobacter pylori* (54).

Within a few years *H. pylori* was recognized as an important factor in the pathogenesis of gastritis and peptic ulcer disease. Interest in this organism has attracted scientists from disciplines such as gastroenterology, microbiology, pathology, immunology, epidemiology and pharmacology. A European Campylobacter (Helicobacter) Pylori Study Group was set up in 1987 in Copenhagen, initiated by Dr S. Gustavsson, Uppsala, Sweden and Dr P. Malfertheimer, Ulm, Germany. During the fourth meeting, in 1991, more than 300 original contributions from all continents were presented, indicating that *H. pylori* has "scientifically infected" the whole world.

Classification of gastritis

Inflammation of the gastric mucosa is the stomach´s response to injury. Increasing numbers of studies are appearing in the literature, documenting the pathology of gastritis and its relation to *H. pylori*. It is now an urgent matter to establish a system for the classification of gastritis that can find widespread acceptance. The classification of chronic gastritis by Whitehead et al. (154), widely used by pathologists, documents mucosal type (antrum or corpus), the grade of gastritis, the activity and the presence of metaplasia.

Strickland and Mackay (140) defined two topographically differing categories of gastritis: Type A and Type B. Type A is chronic atrophic gastritis of the corpus, with parietal cell antibodies and most often a normal antrum. Type B has become synonymous with disease limited to the antrum, and parietal cell antibodies are rare. Correa (18) modified the classification and introduced "autoimmune chronic gastritis" corresponding to type A gastritis, while "hypersecretory gastritis" is similar to type B gastritis.

More recently, Wyatt and Dixon (158) attempted to combine etiology with topography and use the alphabetic nomenclature; type A as gastritis of the autoimmune type and type B representing bacterial gastritis. The different classifications of gastritis mentioned above illustrate the confusing terminology used by pathologists in this field, which makes it difficult to compare histological observations.

In an attempt to find common ground among the above classifications, a new classification of gastritis was devised based on a working party report to the world congress of gastroenterology in Sydney, 1990. The new classification of gastritis is consequently known as the Sydney system (105). This system recognizes three main forms of gastritis: acute, chronic, and "special forms". Morphologically, the following features should be graded: **1**, inflammation; **2**, activity; **3**, atrophy; **4**, intestinal metaplasia, and **5**, presence (density) of *H. pylori*. These variables are graded in a three-grade system (mild, moderate, and severe). Furthermore, the likely etiology should be stated as well as the topographical distribution of the gastritis. Non-graded variables are also included in the system (specific and non-specific). The main compartments of the Histological division of the Sydney system are shown in Fig 1. It is noteworthy that the presence or absence of *H. pylori* is now an important aspect of classification. It is too early to say if this will become the accepted classification in the future.

Diagnostic methods for *H. pylori*

Culture of H. pylori

H. pylori can be cultured by several methods (22, 56, 74, 76, 111, 150, 152). Solid and liquid culture techniques are available with many modifications, especially supplements of whole blood or serum, and antibiotics (85, 107). For primary isolation, a selective medium may be used alone, but most investigators also use a non-selective medium. The media are incubated at 37°C in an atmosphere with 5–7% oxygen, 7% carbon dioxide, 8% hydrogen and 80% nitrogen and a humidity of at least 98% (56). The plates should be examined after 2 up to 5 days.

Biochemical characteristics

H. pylori has a wide spectrum of characteristic features. The strong urease activity of *H. pylori* is used extensively for primary diagnosis and colony confirmation (90, 117), and the findings made during urease investigations in the 1950s and 1960s can now be explained. The

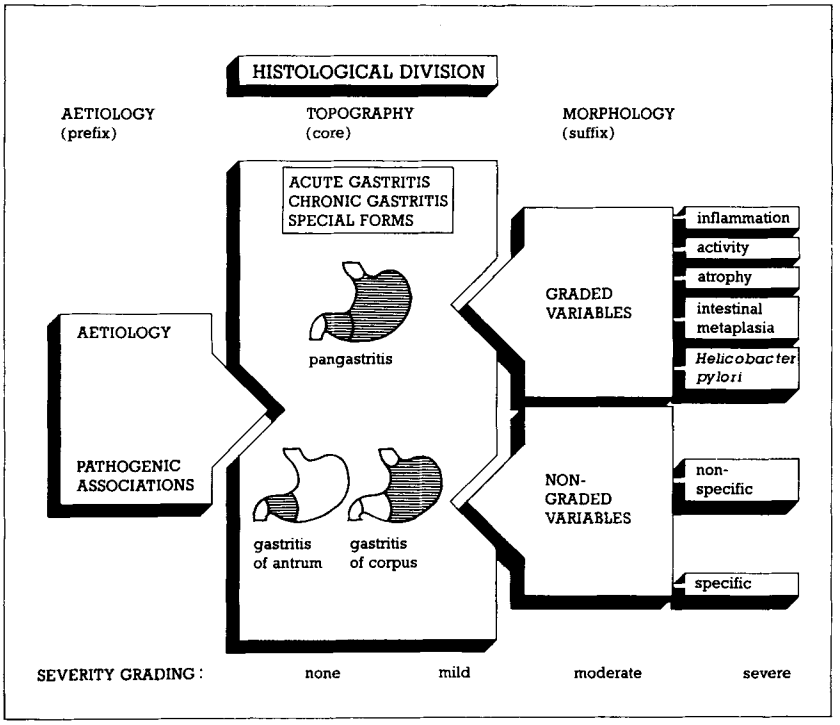

FIG.1 Main compartments of the histological division of the Sydney system for the classification of gastritis. Misiewicz et al. In: World congresses of gastroenterology: working party reports. Melbourne: Blackwell Scientific Publications Ltd., 1990; 1-10.

urease activity of *H. pylori* can be detected with a rapid diagnostic test where a biopsy specimen is inoculated into urease medium and the presence of large numbers of *H. pylori* will lead to a colour change within one hour (67, 103). However, a small number of bacteria will give false-negative results (67). Contamination with other urease producers such as the *Klebsiella/enterobacter* group and *Proteus* spp. will give false-positive results in some cases. This has been confirmed with monoclonal antibodies (MAbs) raised against *H. pylori* and used in an immunofluorescence (IF) test. (32). However, for confirmatory identification of colonies, the urea test is reliable.

H. pylori is oxidase-positive, and produces a large amount of catalase, which can also be used for colony confirmation. Further biochemical reactions may also be used (104).

Histology

H. pylori can be identified histologically in gastric biopsy specimens, by using several staining techniques. Warthin-Starry silverstain (the first described method for the detection of *H. pylori*, 146) is used to demonstrate spirochaetes in tissue (147), but has also proved useful to detect *H. pylori* in the gastric mucosa (102, 146). However, this technique is time-consuming and not specific for *H. pylori*. Other non-specific staining techniques, such as

Gram stain (106, 151), hematoxylin and eosin stain (95), Giemsa stain (60, 123) and acridine-orange staining (145) have been used with varying results. Specific identification of *H. pylori* using monoclonal antibodies has been reported (33). Electron microscopic studies of *H. pylori* colonization has revealed the ultrastructural features of the bacteria as well as mechanisms of pathogenesis (bacterial adherence to epithelial cells, degenerative cellular and intercellular changes). This technique is used mainly in basic research (7, 69).

Serological diagnosis of H. pylori infection

Colonization of the gastric mucosa by *H. pylori* elicits an immune response and several investigators have documented elevated serum antibody levels of specific IgG, IgA and IgM (37, 55, 157). Generally speaking, serological tests are relatively inexpensive. Several different immunological techniques have been tried. Most investigators have used an enzyme-linked immunosorbent assay (ELISA). Depending on the antigen preparation, sensitivity is 80-100% (17, 110). It is important to decide which surface antigens induce specific and dominant antibody responses. A variety of antigen preparations have also been tried, such as sonicated bacteria (71), whole bacterial cells (110) and glycine extracted materia from *H. pylori* (57).

To reduce antigenic cross-reactivity "second generation" ELISAs with purified *H. pylori*-specific antigens have been developed. One important goal has been to avoid cross-reactivity with *C. jejuni* (114).

Urease has been suggested as a specific antigen suitable for use in an ELISA (23). Stacey et al. (138) studied the antigenicity of fractions of *H. pylori* prepared by fast protein liquid chromatography. They found that the purified urease was highly antigenic and that most patients infected with *H. pylori* produce antibodies directed against epitopes on the urease enzyme. Evans et al. (37) used an antigen containing a high molecular weight cell-associated protein, HM-CAP, which possesses urease activity. This antigen was reported to have a specificity of 100% and a sensitivity of 98.7%.

Nucleic acid techniques for detection of H. pylori

A rapid and sensitive system for the detection of *H. pylori* based on nucleic acid has been an attractive alternative to the standard methods described above. Several nucleic acid techniques have been developed to improve the sensitivity. A DNA–RNA hybridization assay using a ^{32}P-labelled synthetic oligonucleotide probe complementary to a nucleotide sequence present in *H. pylori* 16S rRNA was reported by Morotomi et al. (108). Detection of *H. pylori* in stomach tissue by DNA *in situ* hybridization (3), a dot-blot hybridization assay using non-radioactive genomic DNA probes (153) and a molecular cloned DNA probe assay (16) have been reported.

The polymerase chain reaction (PCR) is a technique that amplifies specific DNA sequences (131). Repeated cycles of denaturation, primer annealing and extension carried out with the heat- stable enzyme, Taq polymerase, leads to exponential increases in the target DNA

sequences. The sensitivity of PCR allows the detection of pathogens that would be difficult to identify with conventional techniques.

Detection of *H. pylori* with PCR has been reported from several groups. Hammar et al. (65) amplified a specific 298-bp sequence in a two-step PCR and the sensitivity of the method was 70 bacterial cells visualized on agarose gels. A single primer pair derived from the nucleotide sequence of the urease A gene of *H. pylori* was used in another PCR assay, described by Clayton et al. (15). Valentine et al. (143) amplified target DNA followed by oligoprobe detection of the reaction product. The assay was sensitive for 100 *H. pylori* bacterial cells, but the need for Southern blotting and oligo-probe detection is a drawback. This step was avoided when two groups described the amplification of the *H. pylori* gene encoding 16S rRNA (72, 73). Samples from fresh clinical endoscopic biopsies were amplified and paraffin-embedded gastric biopsies were analysed.

Because of the highly conserved and hypervariable nucleotide-sequence regions, ribosomal RNA sequence has been used for studies on bacterial taxonomy (59). The high copy number of rRNA per bacterial cell also allows enhanced detection sensitivity several thousand-fold greater than DNA–RNA hybridization techniques (148). Also, by including a reverse transcription procedure prior to the PCR reaction, enhanced sensitivity can be achieved.

Animal models for *H. pylori* gastritis

Our understanding of *H. pylori* gastritis would be increased if there were an animal model of the infection for experimental use. Conventionally reared small laboratory animals, such as rats, mice, and guinea pigs, cannot be colonized with *H. pylori* (29). Gnotobiotic animals and small laboratory animals with manipulated immune system are susceptible to the infection (86, 124). In order to assess the susceptibility of an animal model to *H. pylori* infection, several points should be considered (31).

Naturally-occurring ulcers and gastritis in dogs and pigs have been frequently reported (52, 84). When *H. pylori* has been observed in animals it is invariably associated with gastritis. The only animals, so far, known to harbour *H. pylori* seem to be the monkeys *Macaca mulatta* and *M. nemestrina* (9, 115). These organisms found in monkeys are similar to *H. pylori* found by light microscopy, as regards their enzymic, fatty-acid-methyl ester, and protein profiles. *H. pylori* in monkeys are not commensals and seem to be most prevalent in the gastric antrum.

Krakowka et al. (86) and Lambert et al. (88) have described the successful colonization of gnotobiotic piglets with *H. pylori*. The bacteria colonized only the gastric and proximal duodenal mucosa, development of gastritis was observed, and *H. pylori* antibodies were found in serum samples collected at necropsy. The disadvantage with this model and with the gnotobiotic dog model (124) is the difficulty of maintaining the animals in a gnotobiotic condition.

Mucosal immune response

The normal gastric mucosa shows a naive immune status and contains mainly T cells (partly intra-epithelial lymphocytes, IEL), whereas B cells and plasma cells are rare. The gastric epithelium represents a barrier between luminal antigen and the underlying local immune system (5). Complex mechanisms of lympho-epithelial interaction and local immune regulation are involved in the mucosal immune response. When inflammation occurs in the gastric mucosa there is an essential switch from the naive status to processes of an active local immune defence. However, many questions remain unanswered. How are different antigens handled? How does this handling relate to IEL and T cells in the lamina propria (61)? Can epithelial cells present antigen? If so, which mucosal T cell will receive this message (75)? Which are the critical features of microbial–epithelial interactions (155)? Immunologic aspects of *H. pylori* associated gastritis have been reported and speculations about immunopathological effects will be discussed later.

Most persons with *H. pylori* infection produce a circulating antibody response to the organism (116). These circulating antibodies (mainly IgG and IgA) are directed against surface structures of *H. pylori* as demonstrated by immunogold labelling techniques. Despite this antibody response the organism persists in the gastric mucosa. Moreover, the circulating antibodies do not prevent reinfection (89).

Antibody coating (IgG, IgA, and IgM) of *H. pylori* in gastric biopsy specimens from patients with chronic gastritis has been shown using immunoperoxidase techniques (159). Studies on local gastric humoral responses demonstrated specific anti-*H. pylori* antibodies in the gastric juice (126). Short-term organ culture studies showed that *H. pylori*-positive biopsies produced *H. pylori*-specific IgG, IgA, and IgM antibodies, i.e. the gastric juice immunoglobulins are gastric in origin (125). The majority of these locally produced antibodies are of the IgA class.

Chronic gastritis is characterized by an increase in lymphocytes and plasma cells within the lamina propria. According to the Sydney system (105) the severity of the inflammation is graded from mild to severe. These morphological features have become the main characteristics of gastritis. The discovery of *H. pylori* has turned attention to gastric immunity, and in my opinion it is obvious that histological evaluations of gastritis require an immunological basis.

Immunohistochemical staining techniques permit studies of immunohistological patterns in *H. pylori*-associated gastritis. Papadimitriou et al. (119) studied lymphocyte subsets in type B chronic gastritis but did not correlate these findings to the presence or absence of *H. pylori*. They found increased numbers of T cells in both the epithelial compartment and in the lamina propria. The majority of epithelial T cells were found to be of the "suppressor/cytotoxic" phenotype (CD8) whereas CD4 ("helper/inducer" T cells) were more numerous in the lamina propria. Furthermore, they demonstrated HLA-DR antigen expression (see below) on the epithelial cells, indicating that an immunopathologic mechanism is involved in the initiation or maintenance of chronic gastritis. Kirchner et al. (80) extended certain immunological aspects.

The lack of functional analysis of progressing and resolving phases in the local immune response of *H. pylori* render suggestions of immunopathological mechanisms hypothetical.

The pathogenic mechanisms of *H. pylori*

The actual steps leading to infection with *H. pylori* in the gastric mucosa and the induction of inflammation have not been defined. The disease mechanisms are probably both complex and multiple. Newel (113) distinguishes between "colonization factors" and "disease-causing factors".

Motility

The highly motile *H. pylori* possesses numerous flagella. It has been speculated that this motility is essential for *H. pylori* to penetrate the viscous mucin layer and thus avoid gastric acidity (68). Non-motile strains of *H. pylori* do not colonize the stomach of the gnotobiotic pig well, according to one report (27).

Adherence

H. pylori adhere intimately to the gastric epithelium and the adherence is specific for this epithelium *in vivo* (14, 19). Several adhesins have been identified (12, 36, 42) and a surface component of *H. pylori* that interacts with a glycolipid from human erythrocyte membranes has been reported (94). Moreover, *H. pylori* adhere to several types of mammalian cells *in vitro* which does not, however, preclude a role for adherence in pathogenesis (1, 38, 70).

Protection against gastric acidity

In addition to motility as a protective factor against gastric acidity, the strong urease activity of the organism is probably essential for acid protection. Bacterial urease converts endogenous urea into ammonia, which raises the pH of the microenvironment and creates a suitable milieu for the organism (68). Supporting this is that early infection by *H. pylori* is associated with hypochlorhydria (109). Furthermore, it has been speculated that the urease activity could itself be a pathogenic factor (2).

Toxin production

Most *H. pylori* strains produce extracellular proteins that induce intracellular vacuolization of mammalian cells in culture (45). This has also been recognized *in vivo* during experimental infection with *H. pylori* (142), and it has been suggested that the cell damage is caused by toxic mechanisms. Specific *H. pylori* cytotoxins have been characterized. These toxins are not present in strains that do not cause vacuolization (91). Immunoblotting assays with sera from *H. pylori*-infected patients show that cytotoxin-associated polypeptides are immunogenic, and these data suggest that toxins produced by *H. pylori* are important factors in the development

of peptic ulcer (46). However, the mechanism of action of the toxins remains unknown and the significance of these cytotoxins *in vivo* is not known.

Invasion

H. pylori attaches to the surface of the apical epithelial cells, particularly at the tight junctions (69). Electron microscopic studies on biopsies from subjects with gastritis have revealed *H. pylori* within the cytoplasm of epithelial cells inside endocytic vacuoles (7, 136). Evans et al. (40) found that *H. pylori* can be present in the cytoplasm of tissue culture cell lines. The extent of internalization was comparable to that described for other invading pathogens e.g. *Shigella*, enteroinvasive *Escherichia coli* and enteropathogenic *E. coli*.

Immunopathological effects

The local immune response to *H. pylori* has been reviewed above. There are still significant gaps in our knowledge about how the cellular immune response is activated by the organism. However, there are similarities between *H. pylori*-gastritis and other chronic inflammations, such as rheumatoid arthritis.

Heat shock proteins (HSP) are a highly conserved group of proteins produced by prokaryotic and eukaryotic cells in response to a variety of trauma (93). It has become increasingly clear that HSPs play an important role in numerous physiological processes such as unfolding of cytoplasmic proteins and subsequent translocation into the mitochondrion or the endoplasmatic reticulum, and protein assembly of oligomeric protein complexes (78). HSPs serve as important antigens of infectious agents and, perhaps, of autologous transformed cells. Increased synthesis of HSP in pathogens may be caused by stress imposed by the host (78).

HSP 65 kDa is a major antigen of *Mycobacterium leprae*, *M. tuberculosis* and other species of mycobacteria (43). T cells are activated against HSP 65 kDa (53) and data imply that T cells with the γ/δ receptor are specialized to recognize the mycobacterial 65 kDa HSP (8).

RESULTS AND DISCUSSION

H. pylori specific monoclonal antibodies

Monoclonal antibodies were raised against *H. pylori* and the chosen clone, denoted E_7C_{11}, was tested against 140 clinical isolates of the organism with positive results in all cases. It was shown by immunodiffusion that the antibodies were of isotype IgG_1. The antibodies produced by E_7C_{11} reacted with a protein band migrating at approximately 20 kDa as shown by immunoblotting after SDS-PAGE. So far, this MAb has been tested by IF and PAP staining against more than 200 clinical isolates of *H. pylori*, with positive results in all cases (unpublished data, Fig. 2). No cross-reactivity has been observed when tested against a wide panel of unrelated bacteria present in the gut.

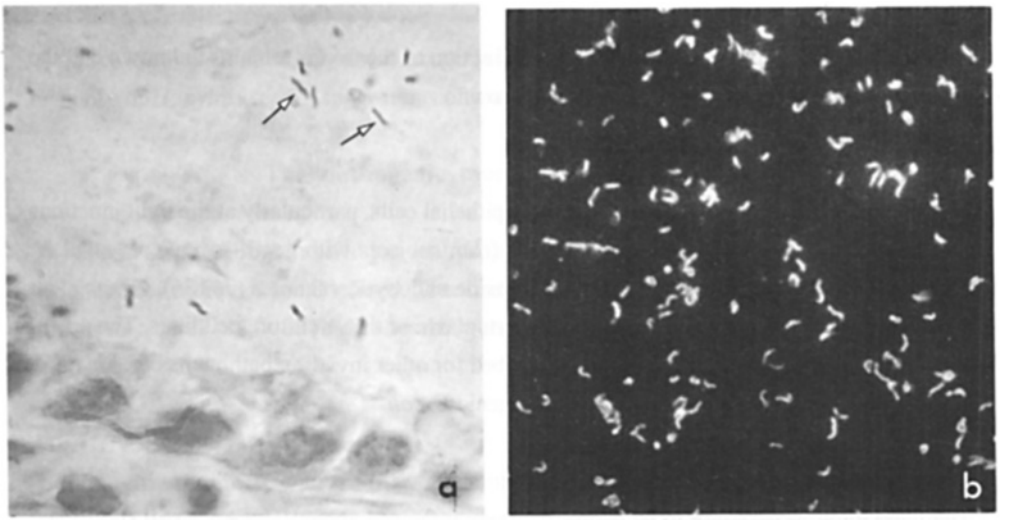

FIG.2 Immunoperoxidase (a) and immunofluorescence (b) staining of *H. pylori* with MAb E_7C_{11} on a frozen section of a gastric biopsy from a patient with gastritis (a) and of cultured cells of *H. pylori* (b).

Immunoperoxidase detection using *H. pylori*-specific MAbs (112) and IF assays using polyclonal antiserum to *H. pylori* (128, 133) have both been reported to be accurate methods for the diagnosis of *H. pylori* infection in biopsy smears and in tissue sections of gastric mucosa. Although these antibodies are available in a limited number of laboratories only, they may serve as an additional tool for the diagnosis of *H. pylori*.

H. pylori and gastric mucosal immunology

It was found that in all gastric biopsies where *H. pylori* could be observed by culturing and immunoperoxidase staining, there was strong expression of class II transplantation antigens on most epithelial cells (Fig. 3). The HLA-DR antigens were the most pronounced, followed by HLA-DP and HLA-DQ antigens. These findings are in agreement with other studies on non-lymphoid cells (49, 144). This indicates that the epithelial cells are theoretically capable of antigen sampling and presentation.

The intestinal epithelium (the gastric epithelium included) constitutes a barrier between luminal antigen and the underlying local immune system. Many factors point to a vital role for the epithelial cells in directing the immune response to luminal antigens; i: their position at the interface between environmental antigens and cells of the mucosal immune system; ii: their relationship to IEL (see below), and iii: their involvement in the transport of secretory IgA and IgM.

In the biopsies where *H. pylori* were found, there was an increase in infiltrating T lymphocytes, with a predominance of CD8-reactive cells in the epithelium and of CD4- reactive T cells in the lamina propria. It is likely that the intestinal epithelium and the T cells of the

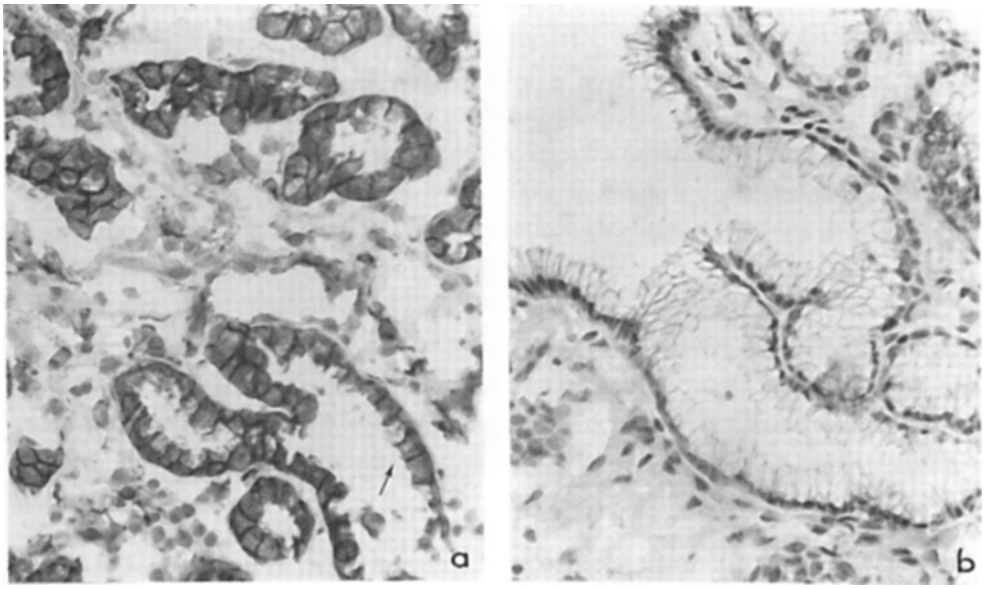

FIG.3 Immunoperoxidase staining of HLA-DR-reactive cells on frozen sections of gastric biopsies from (a) a patient with gastritis and (b) a patient with normal antral mucosa. Note HLA-DR-expressing cells in panel a.

mucosal immune system act in complex. Recently, in patients both with and without *H. pylori* infection, differences in the T lymphocyte phenotypes of cells isolated from the gastric mucosa have been demonstrated by flow cytometry analyzis (10). These results were consistent with those reported above. However, further studies are needed to determine the significance of these observations. One concerns the specificity of the local response to *H. pylori* antigens and the pathogenic relevance and effect of the local immune defence in *H. pylori* colonization.

Local production of γ-interferon by infiltrating T cells may induce expression of HLA-DR on the epithelial cells (134). Factors other than cytokines might, however, be responsible for the induction (82). Little is known about the possible relationship between the handling of different antigens and the epithelial cells with the underlying immune response. In addition to exogenous luminal antigens, auto-antigens may be presented by HLA-DR-positive cells (141).

Evidence that *H. pylori* are capable of intracellular survival (39), in combination with recent observations that stress proteins (HSPs) are major antigens of many pathogens and that T cells with the γ/δ receptor are specialized to recognize such antigens, prompted us to investigate these parameters in *H. pylori*-associated gastritis. We found positive staining of a HSP60 protein (groEL) in the epithelial cells in all *H. pylori* culture-positive biopsy specimens (Fig. 4). In the *H. pylori*-negative specimens, no such staining was observed. Using the γ/δ T cell-specific MAb, we found an increased number of γ/δ T cells located intra-epithelially in biopsy specimens from patients with *H. pylori* -associated gastritis.

Western blotting of *H. pylori* lysates showed that the MAb ML30 reacted with a band migrating at approximately 65 kDa. This protein band was recently purified, and it is now

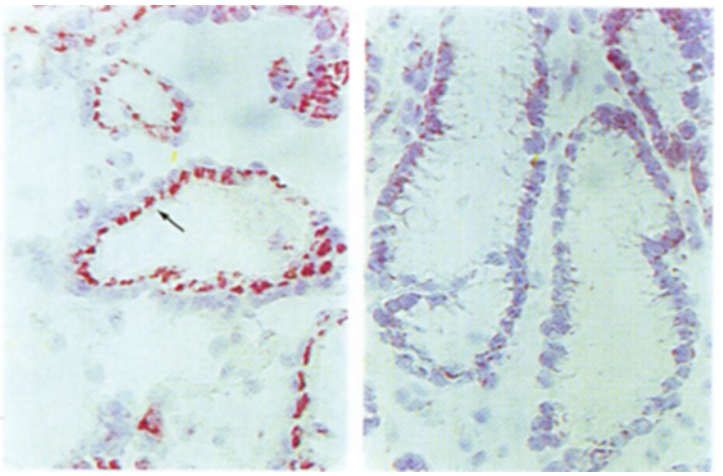

FIG.4 Immunoperoxidase staining of frozen sections of gastric biopsies from (a) a patient with gastritis and (b) a patient with normal antral mucosa. Note presence of the groEL stress protein homologue in epithelial cells (a). In b there is no staining of the epithelial cells.

established that this urease-associated protein (HSP62) is a heat shock protein and a member of the HSP60 family (41).

Although the HSP60 proteins were reported to be mitochondrial proteins (101) present in most mammalian cells, quantitative differences in the expression of epitopes of these proteins may be due to possible expression of "new" HSP60 molecules at intracellular locations. Evidence is now accumulating that *H. pylori* are capable of intracellular survival after endocytosis-mediated uptake *in vitro* and may invade gastric epithelial cells (39). It is possible that HSP62 plays a role in intracellular survival similar to other HSP60 proteins which have been implicated as bacterial survival factors in salmonella (11), chlamydia (13) and mycobacterium infections (25). Another explanation might be that the epithelial cells themselves become stressed by the bacteria, leading to autologous HSP60 expression.

Evidence from murine experimental systems suggests that $CD8^+$ T cells play a role in protection against intracellular bacteria (77). Koga et al. (83) have provided evidence that stressed host cells can serve as targets for murine $CD8^+$ T cells raised against mycobacterial HSP60 peptides. Many groups have shown that γ/δ T cells can be stimulated by mycobacterial antigens (160).

We have only begun to understand the physiological function of these T cells. Not only the number of γ/δ T cells present at the inflammation site but also studies on defined subsets of γ/δ T cells seem to be of importance. Kjeldsen-Kragh et al. (81) demonstrated that in the synovial fluid of patients with inflammatory arthritic diseases the $V\delta1$ subset was predominant and showed a higher activation grade (HLA-DR expression) than the blood-derived $V\delta1$ subset.

The Vδ1 subset was also reported to predominate in the human intestinal mucosa in patients with coeliac disease (64).

The role of the systemic and the local immune response to *H. pylori* calls for further investigation, especially following attempts to eradicate the organism. We were interested in the role of HSP62 in immune reactions to *H. pylori* but, in addition, we wanted to follow certain known though not exhaustively studied inflammatory parameters in patients infected with *H. pylori,* both before and after treatment.

In a treatment study the number of patients in whom *H. pylori* was cleared is shown in Table 1. As seen, already after 2 weeks of treatment, culture results were negative in 8 out of 10 patients. At the follow-up at week 12, 50% (4 of 8 patients) were still culture negative. One additional examination was performed in these cases over a period of 6 to 20 months (Table 1). Cultures from 2 patients remained *H. pylori*-negative at 17 and 6 months, respectively, after the end of treatment.

TABLE 1. Growth of *H. pylori* in gastric biopsy specimens from 10 patients with gastric ulcer and chronic gastritis before and after treatment with Cavedess® and bacampicillin for 6 weeks

Pat. no.	Sex/age	Culture result at week:				
		0	2	4	6*	12
1	M/42	+	-	-	-	+
2	F/61	+	-	-	-	+
3[a]	M/34	+	-	-	-	-
4[b]	M/49	+	-	-	-	-
5[c]	F/64	+	-	-	-	-
6	F/37	+	-	+	-	+
7	F/49	+	+***	+	+	+
8	M/65	+	+	-	-	+
9[d]	M/33	+	-	-	-	-
10	M/64	+	-	+	ND**	+

* End of treatment
** Not done
*** Bacampicillin treatment was concluded
a Culture-pos. at 20 months follow-up
b Culture-pos. at 16 months follow-up
c Culture-neg. at 17 months follow-up
d Culture-neg. at 6 months follow-up

Sera from all *H. pylori*-infected patients were analysed before treatment and we found a strong IgG response to purified urease as well as to purified HSP62 in all cases. Sera from negative controls showed a weak IgG response to both antigens. IgG immunoblots (performed on sera from 4 long-term observed patients) showed that the antibody titres against both antigens decreased after treatment in 2 patients who were culture-negative at that time.

It is clear that HSP62 is a major immunogenic protein in patients with *H. pylori*-associated gastritis. Furthermore, it seems that HSP62 is specific for *H. pylori*. It is not known if these antibodies are auto-antibodies or if complexes of auto-antibodies with auto-antigen can result in maintenance of chronic inflammation in the gastric mucosa.

In the present study no major change in the immunohistological pattern was observed either during or after treatment. The only biopsy specimens that showed neither HLA-DR nor groEL expression on the epithelial cells after treatment were from one patient in whom *H. pylori* was eradicated, i.e. the bacteria could not be cultured either 6 weeks or 6 months after treatment ended.

Lymphocytes must adhere to antigen-presenting cells and antigen-bearing target cells so that normal immunological responses can occur. Lymphocyte function-associated antigen-1 (LFA-1) is a leukocyte cell surface glycoprotein that promotes intercellular adhesion in inflammatory reactions (121). The intercellular adhesion molecule-1 (ICAM-1) is a cell surface ligand for LFA-1 and is inducible on fibroblasts and endothelial cells *in vitro* by inflammatory mediators such as IFN-gamma (26).

ICAM-1 was not present on the epithelial cells, although these cells did express HLA-DR. It has been suggested that ICAM-1 and HLA-DR might be under common regulation, since these antigens are expressed simultaneously in several inflammatory conditions in the skin (62), thyroid (149) and kidney (44). Expression of HLA-DR (but not ICAM-1) on gastric epithelial cells in *H. pylori*-infected individuals raises the question of the mechanisms by which bacterial infection influences expression of intercellular adhesion molecules by these cells and whether epithelial cells in the gastric mucosa express intercellular adhesion molecules other than ICAM-1. We cannot explain why ICAM-1 is not expressed by the epithelium when intra-epithelial LFA-1-positive lymphocytes appear to be present in relatively large numbers (see below). It may be anticipated that other adhesion molecules than ICAM-1 may be involved in the gastric epithelial trafficking of T lymphocytes. It has been shown that another adhesion molecule than ICAM-1 does exist (ICAM-2) and even a third has been suggested (50). ICAM-2 differs from ICAM-1 as regards both distribution and inducibility. Cell-to-cell contact is required in direct cytotoxicity, and lysis of certain targets by T cells appears to occur in an ICAM-1-independent, LFA-1-dependent manner (96)

LFA-1 stained lymphocytes were present within the gastric epithelium as well as in the lamina propria in all *H. pylori*-infected patients. There were more LFA-1 positive lymphocytes than γ/δ T cells. The number of γ/δ T cells in the epithelium varied between the patients and no major change was observed either during or after treatment. Similar findings have been

reported in children with juvenile rheumatoid arthritis where a large number of γ/δ T cells were found in the synovial compartment of some (but not all) children (81).

Could it be that cytotoxic T cells in the gastric epithelium are triggered by immunogenic peptides (i.e. HSP62 from stressed *H. pylori* and/or autologous HSP) present in the gastric epithelial cells, thus initiating a sequence of auto-reactive events due to cross-reactivity through "antigenic mimicry". HSP62 may be the target for intra-epithelial γ/δ T cells cells in a "first line" defence against *H. pylori* and thereafter act as a maintenance factor in the chronic inflammation that will persist for years. The immunopathological effects of *H. pylori* may be caused by stress proteins as has been proposed in other chronic imflammatory diseases (79).

The study of *H. pylori* and gastric mucosal immunology might help us to understand how different parts of this anti-microbial defence system act together. Future studies on T cell response against *H. pylori* antigens as well as studies on *H. pylori* – epithelial cell interactions are needed.

An animal model for H. pylori gastritis

At the age of 8 weeks, 17 barrier-born pigs of Swedish Landrace were studied. These animals are not gnotobiotic, but free from the specific pathogens affecting the individual herds, i.e. they do not harbour certain infectious agents. A clinical strain of *H. pylori* was used for inoculation. Fifteen pigs were inoculated at gastroscopy and 2 served as controls. *H. pylori* could be cultured from 11/15 inoculated pigs. Once the infection was established, the pigs remained culture positive throughout the observation period of 12 weeks. A serum antibody response to *H. pylori* was detectable with ELISA in the infected pigs. Furthermore, focal superficial gastritis developed (Fig. 5). Histological changes, growth of *H. pylori* and specific antibody response to *H. pylori* were not found in the 2 control pigs.

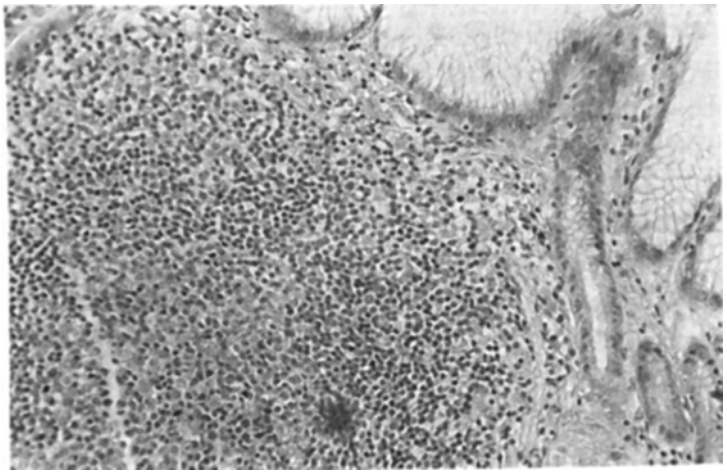

FIG.5 Lymphoid follicle in the lamina propria of a pig inoculated with *H. pylori*.

Two weeks of treatment cleared the organism from the gastric mucosa in 4 treated pigs. The antibody titre remained high after treatment.

H. pylori infection in pigs (whether gnotobiotic or barrier-born) exhibits many of the features of *H. pylori* infection in humans (6, 58). The pig gastric mucosa is similar to the human mucosa regarding the ability of *H. pylori* to adhere and colonize both *in vitro* and *in vivo* (31, 130). Furthermore, experimental *in vitro* studies have shown that viable bacteria in biopsy specimens from humans correlate well with viable bacteria in biopsy specimens from pigs (129). The disadvantage with gnotobiotic pigs is that it is difficult to keep the animals in a gnotobiotic condition. Since *H. pylori*-associated gastritis is a chronic inflammation, the most suitable animal model for this infection is the one that permits long-term studies.

We were able to culture *H. pylori* for up to 6.5 months after the inoculation in barrier-born pigs (35). A topographic mapping of the colonized gastric mucosa was also performed when the pigs were sacrificed. This showed a patchy distribution of the infection, similar to that described in humans.

The barrier-born pig model holds promise for treatment studies since they are more easily performed than in gnotobiotic animals due to the more convenient form of drug administration (no need for a sterile environment). Furthermore, investigations of the pathogenic mechanisms of *H. pylori*-associated gastritis, i.e. bacterial virulence factors such as motility, adhesion, cytotoxic factors and immunopathogenic factors, can now be undertaken (27).

The H. pylori PCR assay

Sequence data of *H. pylori* 16S rRNA were used to select two 22 base oligonucleotide primers. The expected band of 500 bp was amplified from as few as 50 bacterial cells in a PCR assay without the reverse transcription step. The boiling method was efficient when using lysis buffer alone. Thus, the biopsy specimens were homogenized in lysis buffer prior to PCR amplification. When reverse transcription was incorporated into the PCR assay, the sensitivity was further enhanced and we were able to detect as few as two *H. pylori* organisms. No false-positive results from contamination or carry-over were observed.

The PCR assay proved useful for the detection of *H. pylori* in gastric biopsies. There was no inhibitory effect from the colon biopsy tested and we were able to detect as few as ten *H. pylori* cells spiked to the tissue. The amplified fragment expected was easily observed directly in the agarose gel. Moreover, a significant decrease in PCR sensitivity was evident when the reverse transcription step was avoided.

The relative simplicity of this PCR detection method holds promise for extended clinical applications. In contrast to other groups (65, 143) working with PCR assays for *H. pylori* detection, we took advantage of "Nature´s own polymerase-amplified target" (148), i.e. ribosomal RNA present in thousands of copies per bacterial cell. By adding the reverse

transcriptase step to the PCR reaction we were able to recognize a distinct band from two to three bacterial cells without using the time-consuming Southern hybridization.

We decided to target the 16S rRNA gene and not the urease gene because of the greater risk of false-positive results due to amplification of closely related urease-producing bacteria (72). The detection of small numbers of bacteria is important, especially after treatment when coccoid or poorly viable bacteria may be present, even though culture results or breath tests are negative. Furthermore, analysis of environmental samples such as water and suggested *H. pylori* niches such as dental plaques and faeces will probably be reported within a few years. Such samples are currently being investigated with this method.

CONCLUSIONS

- Specific and rapid detection methods for *H. pylori* were developed by raising mouse MAbs. The Mab ($E7C11$) was useful for colony confirmation and for identification of *H. pylori* on frozen gastric biopsy sections when using immunoperoxidase techniques.

- The local immune response to *H. pylori* was studied using immunohistochemical staining techniques. There was an induced expression of MHC class II transplantation antigens (but not of ICAM-1) on the gastric epithelial cells and an increased number of T cells in gastric biopsy specimens from patients with *H. pylori*-associated gastritis. A predominance of $CD8^+$ T cells was found in the epithelium, while $CD4^+$ T cells dominated in the lamina propria. An increased number of T cells expressing the γ/δ receptor was observed within the gastric epithelium in patients with *H. pylori*-associated gastritis. Gastric epithelial cells in patients with *H. pylori*-associated gastritis expressed the groEL stress protein homologue. This homologue (HSP62) was also present in *H. pylori*. These findings support the hypothesis that γ/δ T cells may be involved in host defence and that these T cells may recognize HSP62 expressed both by *H. pylori* and by the epithelial cells.

Patients with *H. pylori*-associated gastritis were treated with a combination therapy and the local and systemic immune responses were studied before and after treatment. *H. pylori* was eradicated from the gastric mucosa in 4 of 10 patients after 6 weeks treatment with Cavedess® and bacampicillin. Long-term follow-up showed reinfection in 2 of these patients. In all patients but one there was no change in the immunohistological pattern either during or after treatment. In this patient in whom *H. pylori* was found eradicated at the long-term follow-up (6 months), there was a normalisation of the local immune response pattern, especially in the epithelial cells.

The humoral immune response to *H. pylori* was studied using immunoblotting and dot blot. It was found that the purified urease and the urease-associated heat shock protein of *H. pylori* (HSP62) are major immunodominant proteins in *H. pylori*. The antibody titres against both

antigens decreased after treatment with Cavedess® and bacampicillin that led to eradication of the organism.

- An animal model for *H. pylori* infection was developed with barrier-born pigs. The infected pigs developed gastritis and a systemic antibody response to *H. pylori* similar to that described for humans. Treatment studies are possible in this model.

- Reverse transcription of 16S rRNA from *H. pylori* followed by PCR-amplification increased the sensitivity of the pathogen detection 50 to 100 fold compared with other PCR assays previously reported (65, 72). *H. pylori* could also be detected with this method in gastric biopsy specimens from gastritis patients.

FUTURE DIRECTIONS

The discovery that *H. pylori* is the major cause of chronic inflammation of the human gastric mucosa has potentially very important implications for treatment and perhaps also for the prevention of peptic ulcer disease. *H. pylori* may also be involved in the initiation of gastric cancer (48, 66, 120). Our understanding of the microbiological and pathogenic aspects of *H. pylori* is continuously being challenged as new results appear from different research areas. In this thesis, I have besides introducing new diagnostic tools, tried to investigate some immunological aspects of *H. pylori*-associated gastritis.

The nature of stress proteins and of the T cell response to them is now the subject of a great deal of interest and speculation. There are a number of fundamental questions in relation to infection and autoimmunity. Several observations suggest that T cells specific for HSP60 are involved in inflammatory diseases such as arthritis (28), and diabetes (30). The concept of molecular mimicry makes it easy to understand the induction of autoimmunity if the bacterial antigen shares reactive antigenic epitopes with tissue components. The possibility that rheumatoid arthritis may be caused by infection is supported by many of the clinical and immunologic characteristics of the disease (137). In human gastritis there is reason to believe that *H. pylori* may be involved in autoimmune pathology due to similarities between the chronic inflammation caused by *H. pylori* and the chronic inflammation found in autoimmune diseases like rheumatoid arthritis where mycobacteria are suggested to play a pathogenic role.

Future research will probably focus on critical features of *H. pylori* – epithelial cell interactions, i.e. the nature of microbial and host receptors, their binding affinity and distribution, and how these interactions trigger the local immune response. More studies on host cell signalling to *H. pylori* (how microenvironmental signals such as temperature, acidity, and phagocytosis influence microbial gene expression) are needed (155). Internalization pathways for *H. pylori* and signals transmitted between bacteria and epithelial cells that govern intracellular processes need to be explored. Finally, the "doorway" across the epithelium,

recently described as M cells by Winner et al. (156), and its relevance for *H. pylori* transport into mucosa-associated lymphoid tissue deserves study.

Strategies for future treatment regimens in *H. pylori*-associated gastritis may also change. Selective immunotherapy in order to modify the reactivity of the gastric immune system may be an alternative way of treatment. Such therapy has been successfully used to block certain critical sites of the CD4 molecule in a patient with systemic vasculitis (100). Another strategy may be to block certain receptors present on *H. pylori* that are responsible for adhesion to the gastric epithelium. Yet another approach would be to block similar receptors on the epithelial cells. However, it is obvious that immunotherapy as well as adhesion inhibition studies on *H. pylori*-associated gastritis require an animal model with which to determine the possibility of using such treatment in humans.

Animal models offer clues to *H. pylori* infection in humans. As mentioned above, one major area of use will probably be treatment studies. *In vitro* activity of an agent against *H. pylori* is no guarantee for *in vivo* success. The development of new drugs in particular requires a convenient animal model for screening purposes, evaluation of side effects and toxic influence of the drug. Investigations of virulence factors and host–*H. pylori* interactions in the early stage of infection will probably engage several research groups from various disciplines.

The PCR technique was developed to provide highly efficient amplification of DNA sequences of interest. Applications of PCR to pathogen detection (including detection of *H. pylori*) have been widely used. The ability to detect *H. pylori* in faeces (72) offers the potential for a non-invasive test for infection. The advantage of detecting low numbers of bacteria may be important to evaluate various antimicrobial regimens. Finally, PCR could be used as an epidemiological tool for the investigation of possible environmental reservoirs of *H. pylori.*

Future directions for *H. pylori* research that will take over or continue from the observations I have reported on in this thesis are mentioned above. However, there are a number of areas of *H. pylori* research that remain unaddressed and open for investigations. There is considerable need for additional work to standardize reagents for the serologic testing of *H. pylori.* We need improved therapy to eradicate *H. pylori* infection. The relatively high frequency of side effects (7–10%) must be reduced if widespread use of anti-*H. pylori* therapy is to be accepted (127). Epidemiology, the natural habitat of *H. pylori*, and mode of transmission of *H. pylori* infection are other areas that have been partly investigated but still need to be addressed and verified.

The acid theory for the pathogenesis of peptic ulcer disease took decades to gain acceptance. *H. pylori* is already the acknowledged cause of chronic gastritis. Within a few years, peptic ulcer and gastric cancer may be included in this statement.

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Correspondence to:

Dr. Lars Engstrand
Department of Clinical Microbiology
University Hospital
S-751 85 UPPSALA
Sweden