

## 4. Other Data Relevant for Evaluation of Carcinogenicity and its Mechanisms

### 4.1 Pathology of infection

Cross-sectional and longitudinal observations in human populations indicate that a series of alterations of the gastric mucosa precede gastric carcinoma (Siurala *et al.*, 1985; Correa *et al.*, 1990b; Kuipers *et al.*, 1994a): They follow a sequential presentation of chronic nonatrophic gastritis, atrophic gastritis, intestinal metaplasia and dysplasia. Atrophy (loss of gastric glands) is a pivotal change in the precancerous process. It radically alters the gastric microenvironment by reducing acid secretion, elevating the gastric luminal pH and resulting in an overgrowth of anaerobic bacteria. Many such bacteria produce reductases which act on nitrate molecules (from food and other sources) and result in elevated concentrations of  $\text{NO}_2^-$  in the gastric lumen. Dietary factors that are important in the progression of the precancerous process include high salt (NaCl) intake and low consumption of fresh fruits and vegetables (Nomura *et al.*, 1982; Fontham *et al.*, 1986; Buiatti *et al.*, 1989b, 1990; Chen *et al.*, 1990; Forman, 1991).

#### 4.1.1 Humans

The anatomical substratum resulting from *H. pylori* infection is chronic gastritis. Although the association between the bacterium and gastritis was recognized only in 1983 (Warren, 1983; Marshall, 1983), the pathological manifestations of chronic gastritis and several nosological entities of gastritis had been described previously.

##### (a) Specific lesions

Colonies of *H. pylori* are characteristically located extracellularly in the mucus layer immediately adjacent to the gastric surface epithelium. They are prominently concentrated in front of the intercellular junctions of the epithelial cells. Most bacteria float freely within the mucus layer; a few adhere to pedestals formed by the epithelial cytoplasmic membrane. They may sometimes penetrate the intercellular spaces and, rarely, the ductules of the parietal cells (Chen *et al.*, 1986; Fiocca *et al.*, 1987; Hessey *et al.*, 1990).

*H. pylori* infection is associated with degenerative changes in the cytoplasm of the surface epithelial cells, identified on haematoxylin–eosin staining as loss of the superficial portion of the cytoplasm, resulting in microerosions of the surface epithelium (Chan *et al.*, 1991). Under the electron microscope, partial loss and stunting of the microvilli and numerous intracellular phagolysosomes may be seen (Chen *et al.*, 1986; Fiocca *et al.*, 1987; Hessey *et al.*, 1990).

*H. pylori* infection results in infiltration of leukocytes into the gastric mucosa. The most abundant are B lymphocytes, which occupy the lamina propria and may lead to formation of

lymphoid follicles (Genta *et al.*, 1993a,b). Polymorphonuclear neutrophils, although less abundant than lymphocytes, are common in *H. pylori* infections. They are seen in the lamina propria, in the space between the epithelial cells and in the gastric lumen; they typically aggregate in the neck area of the gastric glands. Other inflammatory cells identified in *H. pylori*-infected mucosa are plasma cells, T lymphocytes, macrophages and eosinophils (Marshall *et al.*, 1985b; Dixon *et al.*, 1988; Wyatt & Rathbone, 1988; Genta *et al.*, 1993b).

The lesions associated with acute (new) infection are similar to those described above, except that the polymorphonuclear infiltrate is prominent and precedes the lymphocytic infiltrate (Marshall *et al.*, 1985a; Morris & Nicholson, 1987; Graham *et al.*, 1988).

(b) *Nosological entities*

In early stages of *H. pylori* infection, gastritis is nonatrophic. Later, it leads to gland loss (atrophic gastritis) and is frequently followed by intestinal metaplasia.

Atrophic gastritis in patients with the pernicious anaemia syndrome diffusely involves the oxyntic mucosa while sparing the antrum. This gastritis is called type A or autoimmune (Strickland & Mackay, 1973; Correa, 1980). In populations at low risk for pernicious anaemia, atrophic gastritis is multifocal and involves both the antrum and the corpus. This gastritis is called type B (Strickland & McKay, 1973) or multifocal atrophic (Lambert, 1972; Correa, 1980). The two entities coexist in a few patients, leading to the denomination type AB gastritis (Glass & Pitchumoni, 1975). A frequent form of nonatrophic gastritis is located predominantly in the antrum, with mild or no involvement of the oxyntic mucosa. Such lesions have been called diffuse antral (Correa, 1988), interstitial (Cheli *et al.*, 1980), pre-atrophic (Cheli & Testing, 1993) or hypertrophic gastritis (Schindler, 1969). This type of gastritis is seen most frequently in conjunction with duodenal ulcer, while multifocal atrophic gastritis is associated particularly with gastric ulcer or gastric carcinoma (Schindler, 1969; Lambert, 1972).

Once the prominent role of *H. pylori* in chronic gastritis had been recognized, a grading of gastritis, the Sydney system, was designed (Price, 1991), which is intended to include microscopic, gastroscopic and etiological factors, including *H. pylori* infection. The system allows the grading of inflammatory and atrophic changes in the corpus and antrum on a semiquantitative scale of 0–3. The name ‘pangastritis’ is proposed for lesions covering both the antrum and the corpus, which can be atrophic or nonatrophic (Sipponen *et al.*, 1991).

*H. pylori* infection has a prominent role in diffuse antral (nonatrophic) gastritis and in multifocal atrophic gastritis (Siurala *et al.*, 1985). It has no role in corpus-limited (type A or autoimmune) atrophic gastritis, or in other specific forms of gastritis such as those associated with bile reflux or use of nonsteroidal anti-inflammatory drugs, known as ‘reflux’, ‘reactive’ or ‘chemical irritational’ gastritis (Dixon *et al.*, 1988; Flejou *et al.*, 1989), or in ‘lymphocytic gastritis’ (Haot *et al.*, 1986).

*H. pylori* infection has also been associated with other, less frequent types of gastritis, such as that characterized by prominent hyperplastic foveola, also called ‘hypertrophic’ or ‘focal foveolar’ hyperplasia (Stolte *et al.*, 1994b).

(c) *Epidemiology of chronic gastritis*

*H. pylori* infection is very prevalent in some populations of low socioeconomic status (Holcombe, 1992; Sierra *et al.*, 1992). In a few, gastric biopsy specimens and pepsinogen levels indicate that the gastritis is not of the atrophic type (Sierra *et al.*, 1992; Shousha *et al.*, 1993). In populations at high risk for gastric cancer, atrophic forms of gastritis predominate. Atrophic gastritis associated with the pernicious anaemic syndrome, not usually related to *H. pylori* infection, is strongly related to genetic susceptibility and affects mainly populations of northern European extraction. In other populations at high risk for gastric cancer, such as those of the Andean regions of Latin America, those of China and Japan, and US blacks, atrophic gastritis is multifocal and linked in part to dietary factors (Fontham *et al.*, 1986; Nomura *et al.*, 1982).

People of each sex are equally affected, and the prevalence of gastritis is highly age-dependent. Nonatrophic gastritis is more frequent in people under the age of 50, whereas atrophic gastritis and intestinal metaplasia are more frequent among people over that age (Siurala *et al.*, 1985). In samples from 500 blood donors in Finland, the prevalences of both *H. pylori* antibodies (IgG class in particular, but also IgA and IgM) and gastritis were shown to increase with age (Kosunen *et al.*, 1989).

(d) *Relation of infection to gastritis*

The first demonstration of an association between *H. pylori* infection and human disease was the result of two experiments in which *H. pylori* organisms were ingested voluntarily. Acute gastritis was seen in biopsy specimens from both subjects (Marshall *et al.*, 1985a; Momms & Nicholson, 1987), and one of the volunteers developed chronic gastritis. An epidemic of hypochlorhydric gastritis (epidemic achlorhydria) described in 1979 was later shown to be due to transmission of *H. pylori* infection via endoscopy. Acute granulocytic gastritis, lasting some weeks, developed into chronic gastritis within 74 days to two years in these cases (Ramsey *et al.*, 1979; Graham *et al.*, 1988). Successful treatment of *H. pylori* infection leads to healing of gastritis (Rauws *et al.*, 1988; Valle *et al.*, 1991; Kosunen *et al.*, 1992; Genta *et al.*, 1993a).

A positive relationship exists between *H. pylori* infection and gastritis, i.e. with regard to the degree of mucosal inflammation by mononuclear inflammatory cells, polymorphonuclear neutrophils and eosinophils, particularly in the antrum (Stolte *et al.*, 1990; Satoh *et al.*, 1991; McGovern *et al.*, 1991; Stolte *et al.*, 1994b). Specific cytotoxic strains are shown to enhance the inflammatory response, and their occurrence differs between populations.

In a random sample of gastric biopsy specimens from the antrum, corpus or both in Finland, up to 91% of people with nonatrophic (superficial) gastritis, up to 41% with advanced atrophic gastritis but none with normal stomachs or severe atrophic gastritis of the autoimmune (type A, or corpus-limited) type contained *H. pylori* (Siurala *et al.*, 1988). In a subset of patients with advanced atrophic gastritis, the estimated prevalence of *H. pylori* infection was higher when assessed by both serological and histological methods than when it was assessed by histology alone (Karnes *et al.*, 1991). In populations at high risk for gastric cancer, such as in Colombia, the prevalence of *H. pylori* is close to 100% (Correa *et al.*, 1989).

(e) *Atrophic gastritis and intestinal metaplasia*

In a 3–16-year (average, 5.1 years) follow-up study (7290 person-years) of people in Narino, Colombia, the rate of transition from normal histological appearance or superficial gastritis to atrophic gastritis or more advanced lesions was 3.3% per year, corresponding to 1.7% for atrophic gastritis, 0.9% for intestinal metaplasia and 0.7% for dysplasia (Correa *et al.*, 1990b).

Mathematical modelling of cross-sectional data on gastritis in Finland and Estonia indicated a slow, stepwise transition from nonatrophic gastritis to atrophic gastritis over time (Kekki & Villako, 1981; Kekki *et al.*, 1983). The fractional transition rate from the pool of nonatrophic to the pool of atrophic gastritis was estimated to be 2.1–2.6% per year for people aged 25–75 (Kekki & Villako, 1981; Villako *et al.*, 1982).

In an 11.5-year (range, 10–13) follow-up of 113 patients with and without *H. pylori* gastritis in the Netherlands, significant progression of nonatrophic gastritis to atrophic gastritis was demonstrated endoscopically (Kuipers *et al.*, 1994a). Fifteen of 56 (27%) patients with *H. pylori* infection and nonatrophic gastritis developed atrophic gastritis, whereas only two of 49 (4%) patients without *H. pylori* infection, all of whom had normal gastric mucosa at the beginning of follow-up, developed the atrophic stage. The difference was significant ( $p < 0.001$ ).

An endoscopic follow-up of 377 subjects in Finland for 30–34 years (Ihamäki *et al.*, 1985) revealed that progression of atrophic gastritis occurs in the gastric corpus and regression may occur in the antrum in the long term.

Since nonatrophic gastritis involves predominantly the antrum and multifocal atrophic gastritis compromises to a large degree both the antrum and the corpus, it is important to study the dynamics of involvement of *H. pylori* in different regions of the stomach. The location and severity of gastritis vary in different disease manifestations of *H. pylori* infection. Thus, inflammation in duodenal ulcer patients is generally restricted to the antrum, while in those with gastric ulcer and gastric cancer the gastritis is more widely distributed in the corpus of the stomach (Stolte *et al.*, 1990). Observations on patients with different acid outputs may be relevant. In patients given the anti-acid secretory drug omeprazole, gastritis in the antrum is reduced, while inflammation in the corpus increases, i.e. pangastritis is observed (Solcia *et al.*, 1994). In a study on the long-term effects of omeprazole in 91 patients, only 1% had atrophic gastritis at the beginning of therapy, but on follow-up (mean, 48 months; range, 36–64 months), 25% had atrophic gastritis (Klinkenberg-Knol *et al.*, 1994). Other studies of prolonged omeprazole treatment show lower rates of transition to atrophic gastritis (Lambert *et al.*, 1993).

Studies conducted before identification of *H. pylori* also showed changes in the distribution and intensity of gastritis after acid suppression. After vagotomy, a surgical procedure to reduce acid output in duodenal ulcer patients, a marked increase in both the extent and severity of proximal gastritis was seen, but the distal gastritis remained unchanged (Meikhle *et al.*, 1976).

The development of atrophic gastritis depends on factors in addition to *H. pylori* infection (Correa, 1992; Fukao *et al.*, 1993). Genetic susceptibility to atrophic gastritis was seen in segregation analysis in Narino, Colombia, suggesting that expression of a single

autosomal recessive gene, with age-dependent penetrance, is involved (Bonney *et al.*, 1986; see also section 1.1.2(b)).

(f) *Atrophic gastritis and gastric cancer*

In a meta-analysis of six independent follow-up studies (Varis, 1983), 58 cases of gastric cancer (severe corpus-limited atrophic gastritis) were recorded among 843 patients with pernicious anaemia who were followed up for 7.8–15 years (mean, 11 years; 8990 person-years), providing an estimate of 0.6% for the annual cancer risk and suggesting that the occurrence of cancer is approximately five times higher among patients with severe atrophic corpus gastritis than in the population at large. An 11–14-year follow-up of three population samples in Finland (over 800 people) with and without gastritis indicated that the risk for developing gastric cancer was two to three times higher than that expected in people who had advanced atrophic gastritis. All 10 patients with gastric malignancy had had gastritis at the beginning of follow-up, and none without it developed advanced disease (Ihamäki *et al.*, 1991).

Estimates of cancer risk in association with multifocal atrophic gastritis have been based on the results of case-control studies (Sipponen *et al.*, 1985, 1994a), which suggest that the age- and sex-adjusted relative risk for gastric cancer is increased by up to 18 fold. The risk rises to 90 fold in patients with severe pangastric atrophy (Sipponen *et al.*, 1985).

The risk for gastric cancer and, in particular, intestinal-type gastric cancer, is increased in the presence of intestinal metaplasia and atrophic gastritis (Correa, 1992; Sipponen *et al.*, 1992), especially if the intestinal metaplasia is of type III (Jass & Filipe, 1980), also called the colonic or incomplete type (Jass & Filipe, 1979; Jass, 1980; Sipponen *et al.*, 1980). Precancerous lesions of various types and nature (polyps, dysplasia) have been shown to be associated with atrophic gastritis and intestinal metaplasia (Laxén *et al.*, 1983; Correa *et al.*, 1990b). The risk for gastric cancer associated with different types of intestinal metaplasia was investigated in a cohort of 1525 Slovenian patients. The standardized incidence ratio for stomach cancer was 2.2. When type I metaplasia was used as the reference category, the risk was 2.1 for type II and 4.6 for type III (Filipe *et al.*, 1994).

There is some evidence of a relationship between the occurrence of intestinal metaplasia and atrophic gastritis and tumours at the same anatomical site in the stomach (Sipponen *et al.*, 1983).

(g) *Nonatrophic gastritis and gastric cancer*

In a case-control study, the age- and sex-adjusted risk for gastric cancer was slightly but significantly increased (two to three fold) in patients with nonatrophic gastritis over that in subjects with normal, uninfected stomachs (Sipponen *et al.*, 1994).

(h) *Mucosal-associated lymphoid tissue*

B-Cell lymphoid follicles and aggregates resembling intestinal Peyer's patches appearing mainly in the gastric antrum and small curvature of the stomach are a characteristic feature of *H. pylori*-related gastritis; they represent acquired mucosa-associated lymphoid tissue in the stomach (Isaacson, 1992). These follicles do not occur in uninfected subjects or in special forms of gastritis (Stolte & Eidt, 1989; Genta *et al.*, 1993a), whereas they

have been reported to occur in 27–100% of cases with *H. pylori*-related gastritis (Genta *et al.*, 1993a). Their prevalence increases with the degree of inflammatory reaction (Stolte & Eidt, 1989). Treatment of *H. pylori* infection results in a slow decrease (but not the disappearance) of lymphoid follicles within 12 months (Genta *et al.*, 1993b).

In the most comprehensive study, which was designed to determine the frequency and distribution of gastric lymphoid follicles in *H. pylori* infection, mapped gastric biopsy specimens were obtained from 20 normal, uninfected volunteers, 25 asymptomatic volunteers with *H. pylori* infection and no ulcer disease, 21 duodenal ulcer patients, and 16 patients with gastric ulcer. None of the uninfected patients had lymphoid follicles, while all subjects infected with *H. pylori* had follicles. Eradication of the organism with antimicrobial agents resulted in a slow decrease in the prevalence of follicles (Genta *et al.*, 1993a).

#### 4.1.2 *Experimental systems*

Investigation of animals infected with different *Helicobacter* species provides the opportunity to confirm the role of these bacteria in chronic gastritis and also to demonstrate the progression of chronic gastritis to atrophic gastritis.

##### (a) *Non-human primates*

Many studies have shown that a number of primate species are colonized with bacteria similar to *H. pylori*. In a closed colony of rhesus monkeys (*Macaca mulatta*), chronic gastritis was found in 8 of 11 animals surveyed, and inflammation was correlated with the presence of *H. pylori*-like bacteria (Baskerville & Newell, 1988). The inflammatory infiltrate was primarily mononuclear, and the lamina propria was heavily infiltrated by lymphocytes, plasma cells and histiocytes. Large lymphoid follicles occurred in most stomachs. Polymorphonuclear leukocytes were rarely seen. When intense cellular infiltration was present in the body of the stomach, atrophy of glands containing parietal and chief cells was observed.

Examination of another rhesus monkey colony revealed marked abnormalities in a number of animals (Euler *et al.*, 1990). There was a noticeable mixed mononuclear cell inflammatory response in 14/35 animals examined. *H. pylori* was cultured from 12/35 animals. A strong correlation was seen with gastritis: inflammation occurred in 83% of infected animals and in only 17% of uninfected animals. When two groups of five uninfected monkeys without gastritis at the time of screening were inoculated experimentally with either human or monkey isolates of *H. pylori*, the human strain did not colonize the animals, but all of them became infected with the monkey isolate and all had gastritis by 28 days after inoculation.

Dubois *et al.* (1991) found *H. pylori*-like bacteria in 8 of 29 colony-bred rhesus monkeys, and all had gastritis; however, of 14/29 infected with '*H. heilmanni*', only two had gastritis. Uninfected animals had no gastritis.

The Japanese monkey (*Macaca fuscata*) has also been used as an experimental model (Shuto *et al.*, 1993). Of 12 animals inoculated with a human isolate of *H. pylori*, seven became infected and inflammation characterized by polymorphonuclear leukocytes and monocytes was observed. *H. pylori*-associated gastritis persisted in two animals followed for more than 18 months.

(b) *Gnotobiotic piglets*

Krakovka *et al.* (1987) were able to infect 17 gnotobiotic domestic Yorkshire piglets with a human isolate of *H. pylori*. Histopathological lesions indicative of chronic active gastritis were seen in all infected piglets. A neutrophilic response was present for two weeks but then resolved, and the gastritis consisted primarily of mononuclear cells and prominent lymphoid follicles. As piglets can be maintained in the gnotobiotic state for only six weeks, the progression of gastritis could not be assessed.

In a further study, seven pigs were immunized with  $10^9$  *H. pylori* in incomplete Freund's adjuvant in two doses given subcutaneously seven days apart; these pigs and eight unimmunized control pigs were then infected with a human strain of *H. pylori*. The gastritis was much more severe in the previously immunized than in the unimmunized piglets. Neutrophilic infiltrates and neutrophilic gland abscesses were seen in the immunized but not in the unimmunized piglets (Eaton & Krakowka, 1992).

(c) *Dogs*

*H. pylori* has been shown to infect gnotobiotic, germ-free beagle puppies, and significant chronic gastritis was induced in all infected animals (Radin *et al.*, 1990). A more intense gastritis was induced when the pups were inoculated with pure cultures of *H. felis*, an organism commonly seen in dogs (Lee *et al.*, 1992). All infected dogs showed extensive mononuclear inflammation, with the appearance of large lymphoid aggregates. As the animals were kept for only 30 days after infection, no progression of gastritis was observed.

(d) *Cats*

When kittens were infected with either *H. acinonyx*, a species of *Helicobacter* isolated from a group of cheetahs with gastritis, or '*H. heilmannii*', a *Helicobacter*-like bacterium found in the same groups of cheetahs, both organisms colonized the feline stomachs and induced a mild lymphofollicular gastritis, which did not change over 11 months (Eaton *et al.*, 1993).

A closed colony of cats bred by a commercial vendor was shown to be infected by *H. pylori*. The bacterium colonized primarily the antrum and induced antral gastritis (Handt *et al.*, 1994).

(e) *Ferrets*

In a study in which 11 adult ferrets were extensively examined (Fox *et al.*, 1990), *H. mustelae* was present in all animals, and a diffuse antral gastritis similar to that seen in humans infected with *H. pylori* was observed. In some animals, the changes observed in the proximal antrum and the transitional zone appeared to be similar to the early stages of multifocal atrophic gastritis in humans.

(f) *Rodents*

Rodents have not been shown convincingly to become colonized with *H. pylori*; however, the feline *Helicobacter*, *H. felis*, readily colonizes both rats and mice for the life of the animal (Lee *et al.*, 1993). When four-week-old female Swiss-Webster, isolator-reared, axenic mice were given viable *H. felis* orally (Lee *et al.*, 1990), 18/20 mice became infected. The first

evidence of gastritis was seen two weeks after inoculation and was mainly neutrophilic; by four weeks, the severity of inflammation had increased and there were more lymphocytes. By eight weeks, all mice had a relatively diffuse active chronic gastritis, with a cell infiltrate composed of approximately equal numbers of mononuclear and polymorphonuclear leukocytes, with lymphocytes and neutrophils as the predominant cell types. Small lymphoid nodules had formed in the submucosa, and small aggregates of lymphocytes in the subglandular area displaced or compressed mucosal glands. In a more extensive study, the course of gastritis was followed up to 50 weeks after infection (Fox *et al.*, 1993b). Between 20 and 50 weeks, the gastritis became more chronic, although microabscesses were seen in some animals even at this late stage. A similar study in rats showed the induction of chronic gastritis that was less florid than that in the mice (Fox *et al.*, 1991).

The only long-term animal study that allows assessment of the severity of gastritis over the life of infected animals is one in conventional Quackenbush Swiss mice (Lee *et al.*, 1993). A total of 221 seven-week-old female mice were infected with either a living culture of *H. felis* or a gastric homogenate from mice infected with '*H. heilmannii*'. The severity of gastritis was assessed in mice killed at regular intervals for up to 72 weeks. All infected mice showed a slowly progressive chronic gastritis, with increasing numbers of infiltrating mononuclear cells and polymorphonuclear leukocytes. After a year and a half, the inflammatory reaction was so severe that atrophic changes were seen in both the antral and fundic mucosa. Control animals initially showed no inflammatory changes; however, as the animals aged, the gastric mucosa of some animals became infected with a bacterium, *H. muridarum*, that normally inhabits the small and large bowel of the rodent. The presence of this bacterium was also associated with gastritis and atrophic changes.

A severe, long-term gastritis was shown in mice infected for more than six months with an '*H. heilmannii*', *Helicobacter*-like organism originating from a cheetah that had gastritis (Eaton *et al.*, 1993). The infected mice had grossly evident gastric mucosal hypertrophy at sacrifice, with severe lymphoplasmacytic inflammation, lymphoid follicles and microscopic ulcers.

Mice infected with another animal *Helicobacter*, '*Gastrospirillum suis*' from pigs, also developed gastritis (Moura *et al.*, 1993). Some degree of glandular destruction in the oxyntic mucosa due to an inflammatory reaction involving granulocytes and mononuclear cells was described.

## 4.2 Other observations relevant to the interpretation of carcinogenicity and mechanisms of carcinogenesis

### 4.2.1 Humans

*H. pylori* may act in the development of gastric cancer by a number of possible mechanisms: (i) an increase in the rate of epithelial cell proliferation; (ii) damage to mucus secretion and the cytoplasm of foveolar cells; (iii) facilitation of the synthesis and delivery of carcinogens at the site, especially *N*-nitroso compounds; (iv) inhibition of the local effect of antioxidants, especially *L*-ascorbic acid; and (v) induction of mutations and other molecular lesions, either directly or through the release of active oxygen species and NO<sup>•</sup> by polymorphonuclear cells and macrophages attracted by the bacteria.

(a) *Increased cell replication*

Atrophic gastritis increases the rate of proliferation of the gastric epithelium (Lipkin *et al.*, 1985), as measured by tritiated thymidine incorporation. This effect was found to be associated with *H. pylori* infection (Buset *et al.*, 1992; Cahill *et al.*, 1993; Fischbach *et al.*, 1993) in patients with multifocal atrophic gastritis and infected with *H. pylori*. Gastric biopsy specimens taken from patients before and after therapy for *H. pylori* infection were immunostained with antibodies against the proliferating cell nuclear antigen (Brenes *et al.*, 1993). In patients who cleared the infection, the labelling index was reduced from 19.95 to 14.12 ( $p < 0.001$ ), close to the normal index of 13.05. Patients who did not clear the infection showed no reduction in labelling index (18.9 before and 17.9 after treatment). Hyperproliferation of the gastric epithelium thus appears to be caused by *H. pylori* infection.

Both cell proliferation and ploidy have been assessed on the basis of the nucleolar organizer regions. The number of regions is increased in the gastric epithelium of patients infected with *H. pylori*, but after successful treatment the region count is rapidly reduced to normal levels (Correa *et al.*, 1994).

(b) *Alteration of the mucus barrier*

This mechanism is presumed to be important because the gastric microenvironment of atrophic gastritis patients contains concentrations of  $\text{NO}_2^-$  and nitrogen-containing species that can produce carcinogens but may be separated from the target cell by a normal mucus barrier. The gastric epithelium is thus protected from the acid environment in the gastric lumen by complex mucus glycoproteins. *H. pylori* organisms produce proteases and lipases which degrade the mucus gel, causing loss of hydrophobicity (Goggin *et al.*, 1992; Go *et al.*, 1993) and viscosity, which induces breaks in the continuity of the mucus layer (Sidebotham *et al.*, 1991; Slomiany & Slomiany, 1992). This change is followed by increased production of prostaglandin E2 (Oderda *et al.*, 1993). The damage to the mucus is also associated with bile reflux, a common finding in *H. pylori*-associated gastritis (Sobala *et al.*, 1991).

(c) *Facilitation of synthesis of carcinogens in situ*

There is an extensive literature on the possible generation of *N*-nitroso compounds by overgrowing bacteria in the stomachs of patients with atrophic gastritis (Hill, 1986; Correa, 1992). Substrates involved in this process may be nitrogen-containing compounds in foods, which can react with nitrite to produce carcinogenic and mutagenic *N*-nitroso compounds. Examples include indole substances in fava beans (Yang *et al.*, 1984) and Chinese cabbage (Wakabayashi *et al.*, 1985), which are frequently consumed by inhabitants in areas of high risk for stomach cancer. In Costa Rican schoolchildren, *N*-nitrosoproline excretion after proline intake, measured as a marker of endogenous nitrosation, was slightly higher (about 1.5-fold) in an area of high gastric cancer risk than in a low-risk area (Sierra *et al.*, 1993), although *H. pylori* infection is very prevalent (around 70%) in both high- and low-risk areas. These results indicate either that *H. pylori* infection is not causally related to nitrosation or that nitrosation is selectively inhibited in the low-risk area. As *H. pylori* infection is also prevalent in other areas of low risk for stomach cancer, such as in Africa, other environmental, social and genetic factors appear to be involved in the etiology of gastric cancer (Holcombe, 1992).

*H. pylori* contains alcohol dehydrogenase but not aldehyde dehydrogenases. The bacterium can thus produce acetaldehyde from even low (0.1%) concentrations of ethanol (Salaspuro, 1994). Acetaldehyde is a highly reactive, toxic substance which has been classified as possibly carcinogenic to humans by an IARC working group (IARC, 1987).

(d) *Decreased levels of L-ascorbic acid*

Infection with *H. pylori* interferes with the normal capacity of the gastric mucosa to concentrate ascorbic acid. This conclusion is inferred from the fact that uninfected patients have a higher concentration of ascorbic acid in the gastric juice than infected patients (Sobala *et al.*, 1989; Rood *et al.*, 1994); furthermore, previously infected patients can concentrate ascorbic acid at near normal levels after successful antimicrobial therapy (Sobala *et al.*, 1993; Ruiz *et al.*, 1994).

(e) *Induction of mutations*

*H. pylori* has no direct mutagenic activity, and reports of differences in the mutagenicity of gastric juice from patients with and without gastritis are equivocal (Montes *et al.*, 1979; Morris *et al.*, 1984; O'Connor *et al.*, 1984; Farinati *et al.*, 1989). Investigations of alterations in the *p53* gene in 10 gastric adenomas and one carcinoma, however, showed that three of the adenomas contained *p53* mutations (Tohdo *et al.*, 1993). In a study of samples obtained by gastrectomy from 12 patients with gastric cancer in Italy, mutations of the *p53* gene were found in 3/12 normal areas of the stomach, 4/8 areas of metaplasia, 8/12 areas of dysplasia and 9/12 of the carcinomas. In five of seven of the samples that were analysed further, the mutations were shown to be GC→AT transitions in exons 5–8 (Shiao *et al.*, 1994). Amplification of the *C-erbB.2* gene is related to invasion and nodal involvement. Differential expression of the ras oncoprotein in diffuse-type and in poorly differentiated intestinal-type gastric carcinomas implies that there are two distinct subtypes of gastric carcinoma (Tehara, 1993).

It has been proposed that the activation of polymorphonuclear leukocytes that occurs in the gastritis induced by *H. pylori* could result in the production of oxygen and nitrogen radicals (e.g. hydroxy radicals, nitric oxide), which induce DNA damage (Wink *et al.*, 1991; Nguyen *et al.*, 1992). Davies *et al.* (1994) reported that gastric biopsy specimens from *H. pylori*-infected subjects show more production of reactive oxygen metabolites than specimens from uninfected individuals. An inducible form of nitric oxide synthetase was detected immunohistochemically in epithelial cells of the stomach infected with *H. pylori* in subjects with chronic atrophic gastritis (Pignatelli *et al.*, 1994). [The Working Group noted the inadequate reporting of the data.]

(f) *Cytotoxin and cytotoxin-associated protein*

Only one, variable property of *H. pylori* has been shown to be correlated with the severity of disease. It is the vacuolating cytotoxin, first described by Leunk *et al.* (1988), who showed that broth-culture filtrates induced intracellular vacuolation in seven of nine mammalian tissue culture cell lines tested. This toxin was later found to be an 87-kD protein with partial homology with the internal sequences of ion channel proteins (Cover & Blaser, 1992).

Bacterial culture filtrates from a cytotoxin-producing strain of *H. pylori* were incubated with cell cultures. After 16 h, cells were harvested and the Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was

measured. An immediate reduction in enzyme activity was observed. Filtrates of a non-cytotoxin-producing strain did not inhibit enzyme activity (Ricci *et al.*, 1993).

Soon after identification of the cytotoxin, it was shown that a greater proportion of strains isolated from duodenal ulcer patients were toxigenic (66.6%) than strains isolated from asymptomatic patients (30.1%) (Figura *et al.*, 1989). Also, patients with duodenal ulcer were more likely to have antibodies that neutralize the activity of the toxin in their serum than asymptomatic patients (Pereira Lage *et al.*, 1993).

The sera from all six gastric carcinoma patients and three of five sera from peptic ulcer patients showed neutralizing activity to the cytotoxin, and 21 of 22 stored sera from gastric cancer patients also showed neutralizing activity (Hirai *et al.*, 1994). [The Working Group noted the small number of sera in the first part of this study and the lack of control sera from non-cancer patients in the retrospective study.]

In a study of 30 *H. pylori* patients, 47% of the infecting strains were toxin producers. Cytotoxin production *in vitro* was shown to be associated with increased antral mucosal polymorphonuclear leukocyte infiltration (Cover *et al.*, 1993). Sixty-nine percent (18/26) of strains of *H. pylori* isolated from patients with diffuse antral gastritis and 89% (70/79) of strains isolated from patients with chronic atrophic gastritis were toxin producers ( $p = 0.043$ ) (Fox *et al.*, 1992).

Antibodies against an *H. pylori* 120-kDa protein were found in gastric biopsy specimens from patients infected with *H. pylori*. The presence of the antibody was correlated strongly with the presence of peptic ulcer and severe gastritis (Crabtree *et al.*, 1991). This very immunogenic protein is expressed in association with the vacuolating toxin (Crabtree *et al.*, 1992), and the antigen has been named *cagA* (cytotoxin-associated protein); its gene (*cagA*) has been sequenced. Clinical isolates that do not produce the antigen do not have the gene and are unable to produce an active vacuolating cytotoxin (Covacci *et al.*, 1993). An ELISA for the 120-kDa protein on sera has allowed investigations of the sera of *H. pylori*-infected patients (Crabtree *et al.*, 1992).

Crabtree *et al.* (1993a) examined the systemic IgG response to *H. pylori* in 70 gastric cancer patients; 79% were seropositive by ELISA for *H. pylori* infection. Of these ELISA-positive sera, 91% recognized the *H. pylori* 120-kDa *cagA* protein by western blotting, significantly more than a control group of 47 ELISA-positive patients with non-ulcer dyspepsia (72%).

Cytotoxic strains that express the *cagA* antigen of *H. pylori* have also been shown to induce rapid secretion of significantly more IL-8 in gastric epithelial cell lines than non-cytotoxic strains (Crabtree *et al.*, 1994). IL-8 has been shown to be expressed *in vivo* in *H. pylori*-infected people and is known to be a potent neutrophil chemotactic and activating factor (Crabtree *et al.*, 1993b; Noach *et al.*, 1994). Increased IL-8 production has also been seen in neoplastic tissue. In a further study using immunofluorescence techniques to locate IL-8 in cryosections of gastric and duodenal biopsies and resected gastric tumour tissue samples, it was found in the epithelium of histologically normal gastric mucosa, with particularly strong expression in the surface cells. Gastric epithelial IL-8 expression was increased in chronic *H. pylori*-associated gastritis, and expression of IL-8 within the lumina propria was evident. Gastric carcinoma cells also expressed IL-8 (Crabtree *et al.*, 1994).

#### 4.2.2 *Experimental systems*

Molecular lesions, cell changes and other precancerous markers have not been measured directly in experimental animals, but *Helicobacter*-induced changes have been mimicked and the effects measured. Thus, Tsujii *et al.* (1993) administered ammonia to rats in the drinking-water for three days a week for one, two, four and eight weeks at a concentration (0.01%) that was considered to be equivalent to that of gastric juice in *H. pylori*-infected people (reported to be 0.015%, as compared with < 0.005% in uninfected people) (Triebeling *et al.*, 1991; Neithercut *et al.*, 1993). Controls were given tap-water alone. After four to eight weeks, the mucosal thickness of the antrum but not of the body of the stomach was decreased. Epithelial cell migration rates, measured by incorporation of 5-bromo-2'-deoxyuridine (BrdU), were significantly increased, particularly in the antrum. The BrdU-labelling index was also significantly increased in all ammonia-treated groups. The proliferative zone in the antrum was significantly enlarged as mucosal atrophy developed, whereas in the corpus mucosa enlargement of the proliferative zone occurred despite the absence of mucosal atrophy.

In an investigation by the same group of the possible role of ammonia as a promoter, 85 male Sprague-Dawley rats, five weeks of age, received MNNG at 83 mg/L in the drinking-water for 24 weeks. Forty treated animals were then given tap-water, and 40 were given 0.01% ammonia in the drinking-water. Animals were kept for a further 24 weeks. All rats were killed when moribund or at 48 weeks after the commencement of MNNG treatment. A significantly higher proportion of the rats given MNNG followed by ammonia developed gastric adenocarcinomas (26/37) than those given MNNG followed by tap-water (12/39;  $p < 0.01$ ) (Tsujii *et al.*, 1992).

Administration into the stomachs of mice of a sonicated sample of a cytotoxin-producing strain of *H. pylori* induced epithelial vacuolation and limited infiltration of mononuclear cells into the lamina propria. A sonicated sample of a non-toxin-producing strain did not cause epithelial lesions. The *H. pylori* cytotoxin gene has been cloned into *E. coli*, where a protein was synthesized as a 140-kDa precursor that is processed to a 94-kDa fully active toxin. Oral administration of this recombinant toxin to the mice induced vacuolation but not cell infiltration (Telford *et al.*, 1994).

Consistent with the observation of changes in the location of gastritis with reduced gastric acidity, *H. felis*, which is normally restricted to the antrum, appears in the body of the stomach of rodents given the acid-suppressive drug, omeprazole. Groups of specific pathogen-free BALB/c mice were colonized with *H. felis* and given omeprazole or no treatment for one month; one month after cessation of treatment, *H. felis* was seen in all areas of the stomach in the omeprazole-treated group but only in the cardia and antrum in the controls (Danon *et al.*, 1994). A similar result was obtained in omeprazole-treated *H. felis*-infected rats (Mellgard *et al.*, 1994). [The Working Group noted the incomplete reporting of the data.]

A recently identified bacterium, *H. hepaticus*, was first isolated in association with hepatocellular tumours in mice. Mice infected with this bacterium developed liver lesions, but tumour development has not yet been seen because of the short duration of the experiments reported (Ward *et al.*, 1994).