Helicobacter pylori infection in a mouse model: Development, optimization and inhibitory effects of antioxidants

Wang, Xin

2001

Helicobacter pylori infection in a mouse model

Development, optimization and inhibitory effects of antioxidants

Xin Wang

LUND UNIVERSITY

2001

Helicobacter pylori infection in a mouse model

Development, optimization and inhibitory effects of antioxidants

Akademisk avhanding
som med vederborligt tillstånd av Medicinska fakulteten vid Lunds Universitet for avlaggande av doktorsexamen i medicinsk vetenskap kommer att offentligen forsvaras i Rune Grubb Salen, BMC, Solvegatan 19, Lund, Fredag den 12 Oktober 2001, kl. 13.15

Xin Wang

LUND
UNIVERSITY

Fakultetsopponent: Associate professor Elisabeth Norin, Microbiology and Tumor Biology Center (MTC), Karolinska Institutet, Stockholm

Organization
LUND UNIVERSITY

Department of Medical Microbiology, Dermatology and Infection
Sölvegatan 23,223 62 Lund

Document name
DOCTORAL DISSERTATION

Dare of issue
October 12,2001

Sporisoring organization

Author(s)
Xin Wang

Title and subtitle

Helicobacter pylori infection in a mouse model: Development, optimization and inhibitory effects of antioxidants

Abstract

Heficobacterpyfori is a human pathogen strongly associated with chronic type B gastritis, peptic ulcer disease, gastric cancer and MALT lymphoma. Our aims were to establish a mouse model of ***H. pylori*** infection, to study pathogenesis of gastritis and gastric cancer and to investigate new treatment strategies in this model. Both spiral and coccoid forms of *H.* ***pyfori*** strains 17874, 25 and 553/93 caused chronic gastritis in BALB/cA mice during 30 weeks of infection. Infection with strain 553/93 displayed the most severe gastritis. Specific antibodies were detected using ELISA and immunoblot in mice infected with ***H. pylori*** following 4 weeks for 17874 spirals, and 16 weeks for 17874 coccoids, and both sprials and coccoids of two other strains. Dietary factors influenced the abilities to recover ***H. pyfori*** from the infected mice through culture. One diet, without fish meal and heated at 140-150°C, gave the optimal *H. pylori* colonization and highest inflammation score in murine stomach among 4 commercial rodent diets tested. The changes were most dramatic (100% to 10-20% infection rate) when the infection was carried out in animals already fed on a specific diet. A *H.* ***pylori*** *strain* 119/95 (CagA and VacA positive) was found to predominate (90.5%) in the murine stomach from inocula containing nine *H.* ***pyfori*** strains by RAPD-PCR among 577 colonies recovered from mice. C57BL/6 and BALB/cA mice showed higher inflammation scores than CBA/Ca or NMRI mice at 4 and/or 10 weeks post-inoculation. Subsequently C57BL/6 mice (n=5) inoculated with a *H.* ***pylori*** mouse passaged strain 119195 developed a gastric squamous cell papilloma after 13 months. Three out of five animals developed high-grade B-cell lymphoma derived from a MALT lymphoma at the squamous-corpus border with manifestations also in the liver, spleen and kidney. ***An*** astaxanthin-rich algal meal from the microalga *Hamatococcus pluvialis* and vitamin C inhibit *H.* ***pylori*** growth at 0.3125 to 2.5 mg/ml (astaxanthin content, 6.25 to 50 μg/ml) and 0.5 to 2 mg/ml ***in vitro*** respectively. Furthermore, these antioxidants decreased the gastric colonization and inflammation significantly in infected BALB/cA mice. The effect on inhibition of *H.* ***pylori*** infection by the astaxanthin-rich algal meal was found to occur simultaneously with a modulation *H. pylori*-induced T-lymphocyte response, switching from a Th1- to a Th1/Th2-response. These studies illustrate that *H.* ***pyfori*** can induce chronic gastritis and gastric lymphoma in an optimized mouse model. Dietary factors influence *H.* ***pylori*** infection and antioxidants may become a new treatment strategy against *H.* ***pylori*** infection in humans.

Key words

Yelicobacterpylori, mice, gastritis, lymphoma, antioxidant, diet, cytokines and Thl and Th2

Classification system and/or index termes (if any)

Supplementary bihliographical information:

Language
English

ISSN and key title

ISBN
91-628-4864-X

Recipient's notes

Number of pages
128

Price

Security classification

Distribution by (name arid address)
Xin Wang, Dept. of Med. Microbiol., Dermatol. & Infect., Lund University, Sölvegatan 23, S-22362 Lund, Sweden

Signature Xin Wang Date August 27, 2001

Helicobacter pylori infection in a mouse model

Development, optimization and inhibitory effects of antioxidants

Xin Wang

Department of Medical Microbiology,
Dermatology and Infection
University of Lund
Sweden

LUND
UNIVERSITY

2001

ISBN 91-628-4864-X

To Daiqing, Yinuo and

my parents

Contents

List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-VI. Reprints were made with permissions from the publishers.

I. Wang X., E. Sturegård, R. Rupar, H. O. Nilsson, P. A. Aleljung, B. Carlen, R. Wíllén, and T. Wadström. Infection of BALB/cA mice by spiral and coccoid forms of *Helicobacter pylori*. J Med Microbiol. 1997;46:657-663.

II. Wang X., H. Sjunnesson, E. Sturegård, T. Wadström, R. Willén, and P. Aleljung. Dietary factors influence the recovery rates of *Helicobacter pylori* in a BALB/cA mouse model. Zent bl Bakteriol. 1998;288:195-205.

III. Wang X., R. Willén, T. Wadström, and P. Aleljung. RAPD-PCR, histopathological and serological analysis of four mouse strains infected with multiple strains of *Helicobacter pylori*. Microbial Ecology in Health and Disease. 1998;10:148-154.

IV. Wang X., R. Willén, C. Andersson and T. Wadström. Development of high-grade lymphoma in *Helicobacter pylori*-infected C57BL/6 mice. APMIS 2000;108:503-508.

V. Wang X., R. Willén and T. Wadström. Astaxanthin-rich algal meal and vitamin C inhibit *Helicobacter pylori* infection in BALB/cA mice. Antimicrob Agents Chemother. 2000;44:2452-2457.

VI. Bennedsen M., X. Wang, R. Willén, T. Wadström and L. P. Andersen. Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. Immunol Lett 1999;70:185-189.

Abbreviation

***cag*A**	Cytotoxin-associated gene A
CagA	Cytotoxin associated protein
CAT	Catalase
ELISA	Enzyme linked immunosorbent assay
GSH	Glutathione
H. pylori	*Helicobacter pylori*
Helicobacter spp.	*Helicobacter* species
HHLO	*Helicobacter heilmannii*-like bacteria
IFN	Interferon
IL	Interleukin
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NUD	Non-ulcer dyspepsia
MALT	Mucosa-associated lymphoid tissue
MNU	N-methyl-N-nitrosourea
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
p.i.	Post-inoculation
RAPD-PCR	Random Amplified Polymorphic DNA-polymerase chain reaction
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SPF	Specific pathogen free
Th	T-cell helper
TNF	Tumor necrosis factor
***vac*A**	Vacuolating cytotoxin gene A
VacA	Vaculating cytotoxin A
VBNC	Viable but non-culturable

Introduction

The history of Helicobacter pylori

The presence of spiral-shaped bacteria have been reported in studies including human patients since 1889, but the organism *Helicobacter pylori* was discovered almost one hundred years later [Kidd *et al.* 1998]. The organism associated with active chronic gastritis was isolated in 1983 and named *Campylobacter pyloridis* in 1984 [Warren *et al.* 1983; Marshall *et al.* 1984]. It fulfilled Koch's postulates in one healthy volunteer [Marshall *et al.* 1985]. The name was first changed to *Campylobacter pylori*, then to *Helicobacter pylori* in 1989 when taxonomic studies showed that this organism did not belong to the genus *Campylobacter* [Goodwin *et al.* 1989].

Helicobacter pylori (*H. pylori*) infections are ubiquitous and causes chronic gastritis, peptic ulcer and gastric carcinoma [Hansson *et al.* 1996; You *et al.* 2000; Woodward *et al.* 2000]. Many different animal species are infected by their own *Helicobacter* species other than the human pathogen *H. pylori*, which suggests common ancestors for these organisms colonizing the gastric mucosa, intestines and liver (Table 1).

Microbiology

H. pylori is a Gram-negative spiral shaped or curved rod microbe, 2-5 μm long, 0.5-1 μm wide, with 1-6 unipolar flagella (Fig. 1). *H. pylori* grows in a moist and microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) at 37^{o}C. A variety of solid culture media were developed, usually including 5-10% horse or sheep blood [Hachem *et al.* 1995]. Colonies are usually visible after 3-5 days, but may need 7 or more days. Broth can also be used and selective antimicrobial agents can be added to inhibit growth of contaminating bacteria or yeast. *H. pylori* is a urease, oxidase and catalase positive organism.

Spiral forms of *H. pylori* convert to coccoid forms (Fig. 2) under conditions of nutrient starvation, antibiotics, oxygen excess, and other stress factors

Table 1. Isolated *Helicobacter* species, their hosts and diseases [Fox *et al.* 1997; Ferrero *et al.* 2001a].

Species	**Hosts**	**Experimental hosts**	**Tissue sites**	**Disease(s)**
H. pylori	Human, macaque, cat	Mouse, piglet, monkey, cat, gerbil, guinea pig	Stomach	Gastritis ± ulcer
H. mustelae	Ferret, mink	Ferret	Stomach	Gastritis ± ulcer
H. felis	Cat, dog	Mouse, dog, rat	Stomach	Gastritis
H. heilmannii [a]	Dog, cat, monkey, swine, human,	Mouse	Stomach	Gastritis ± ulcer
H. bizzozeronii [a]	Dog, human	-	Stomach	-
H. nemestrinae	Pig-tailed macaque	-	Stomach	-
H. suis	Swine	-	Stomach	-
H. acinonyx	Cheetah	Mouse	Stomach	Gastritis
H. salomonis	Dog	-	Stomach	-
H. aurati	Hamster	-	Stomach, intestine	-
H. westmeadii	Human	-	Intestine	-
H. mesocricetorum	Hamster	-	Intestine	-
H. rappini	Sheep, dog, human, mouse	Guinea pigs	Stomach, intestine, liver	Abortion Hepatic necrosis
H. canis	Dog, human	-	Intestine, liver	Gastroenteritis Hepatitis
H. hepaticus	Mouse	Mouse	Intestine, liver	Hepatitis Hepatocarcinoma
H. bilis	Mouse, rat, dog	Mouse	Stomach, intestine, liver	Hepatitis
H. rodentum	Mice	-	Intestine	-
H. trogontum	Rat	-	Intestine	-
H. muridarum	Mice, rat	-	Stomach, intestine	-
H. cinaedi	Human, hamster	Macaque	Intestine	Proctitis Colitis Hepatitis
H. fennelliae	Human	Macaque	Intestine	Proctitis Colitis
H. pullorum	Human, chicken	-	Intestine, liver	Hepatitis
H. pametensis	Bird, swine	-	Intestine	
H. cholecystus	Hamster	-	Liver	Cholaniofibrosis Centrilobular Pancreatitis

[a]: closely related, maybe same species; -: not described.

[Donelli *et al.* 1998; Mizoguchi *et al.* 1999; Nakamura *et al.* 2000]. The coccoid form of *H. pylori* fails to grow on the routine bacteriological media, but animal passage or nutrient supplementation may help to recover. The viable but non-culturable (VBNC) hypothesis showed that at least 30 species contained in 16 genera of mostly gram-

negative bacteria [Oliver 1995], and was proposed for *H. pylori* [Bode *et al.* 1993]. Animal passage of *H. pylori* coccoids was reported to revert in mice [Cellini *et al.* 1994], but not in gnotobiotic piglets or upon a passage in eggs [Eaton *et al.* 1995; Enroth *et al.* 1996]. These coccoid forms are able to attach to gastric epithelial cells and induce the same cytoskeletal changes and possess about the same proteins as spiral forms suggesting they are viable [Zheng *et al.* 1999; Nilsson *et al.* 2000; Willen *et al.* 2000; Sisto *et al.* 2000]. In a recent review the coccoid forms of *H. pylori* have been proposed to exist in three forms—a dead degenerative form, a viable but non-culturable form, and a viable and culturable form—which seem to have genetic and biochemical properties that are nearly identical to those of the spiral form [Andersen *et al.* 2000]. The importance of coccoid forms of *H. pylori* is under debate with regard their role to both transmission and treatment failure [Cave 1997; Cellini *et al.* 2001].

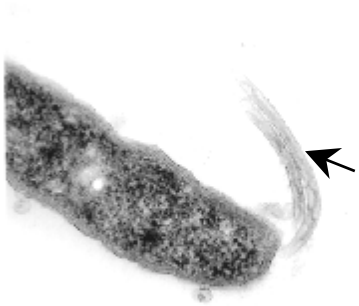


Fig. 1 *H. pylori* vegetative cell
Note flagella (arrow).
(Photo from Roger Willén).

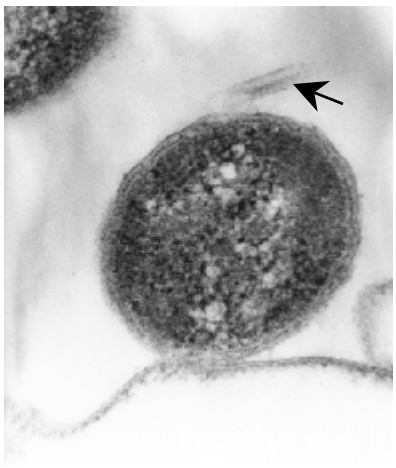


Fig. 2 *H. pylori* coccoid form
Note flagella (arrow).
(Photo from Roger Willén).

Epidemiology and transmission

Approximately 60% of the world's population is infected with *H. pylori* [Nomura *et al.* 1991; Marshall 1994]. Although this infection is worldwide, it is less prevalent in Western Europe, where approximately 30% of the population is infected, and more prevalent in other parts of the world, where more than 90% of the population is infected [Malaty *et al.* 1992; Marshall 1994]. This difference in prevalence of infection has been attributed to the rate of acquisition of *H. pylori* in childhood [Mitchell *et al.* 1992]. Several studies have been shown a correlation with

low socioeconomic status and a high rate of *H. pylori* infection [Mendall *et al.* 1992; Malaty *et al.* 1994; Woodward *et al.* 2000].

Despite the worldwide spread of *H. pylori* infection, the route of transmission is still unknown. Person-to-person transmission either by the gastro-oral, oral-oral or fecal-oral routes is considered to be the most probable mode [Desai *et al.* 1991; Thomas *et al.* 1992; Luzza *et al.* 2000]. A zoonotic transmission has been disputed [Handt *et al.* 1994; Grubel *et al.* 1998; Bode *et al.* 1998; Osato *et al.* 1998]. A positive association between the prevalence of *H. pylori* in Sardinian shepherds and contact with sheep and sheepdogs is reported [Dore *et al.* 1999a] and suggested that "the cycle of *H. pylori* infection might, in certain circumstances, include phases in the environment, animals (sheep or dogs) and human beings" [Dore *et al.* 1999b].

Pathogenic mechanisms of H. pylori

Virulence factors

The main characteristics of bacterial virulence factors are: 1) to facilitate colonization of the host; 2) to enable the pathogen to avoid host defense mechanisms and 3) to cause tissue damage of the host. A number of virulence factors of *H. pylori* have been well defined (Table 2). By flagella movement, *H. pylori* is able to penetrate the gastric mucus layer thereby escaping the acidic environment in the lumen and approaching the gastric mucosal surface. The colonizing *H. pylori* adhere to gastric epithelium by different bacterial adhesins using hemagglutinins, laminin and Lewis b antigens as receptors. High urease production increases the pH and activates the host immune defense.

Immune response to *H. pylori*

Despite an apparently vigorous inflammatory response against *H. pylori*, most infected people fail to clear the pathogen spontaneously [Ernst 1999]. A humoral response to *H. pylori* lead to the production of antibodies (mainly IgG and IgA) to cell surface and soluble products used for serodiagnosis [Nilsson *et al.* 1997]. *H. pylori* elicits a network of cell-mediated effector mechanisms, including various inflammatory cells and their cytokines. The nature of cytokines produced by the host in response to *H. pylori* depends on several factors, including the genetic background.

Table 2 *H. pylori* virulence factors and their effects [Andersen *et al.* 2001].

Bacterial properties	Virulence factors	Effects/properties
Colonizing	Flagella	Active movements through mucin
	Urease	Neutralization of gastric acid
	Adhesins	Anchoring *H. pylori* to the epithelium
Tissue damaging	Proteolytic enzymes	Glucosulfatase degrades mucin
	120-kDa CagA	Related to ulcer and severe gastritis
	VacA	Damage of the epithelium
	Urease	Toxic effect on epithelial cells, disrupting cell tight junctions
	NAP (neutrophil activating protein)	Lead to neutrophil-mediated mucosal injury
	Phospholipase A	Digest phospholipids in cell membranes
	Alcohol dehydrogenase	Gastric mucosal injury
Survival	Intracellular surveillance	Prevent killing in phagocytes
	Superoxide dismutase	Prevent phagocytosis and killing
	Catalase	Prevent phagocytosis and killing
	Coccoid forms	Dormant form
	Heat shock proteins	Sheathing antigen
	Urease	Prevent phagocytosis
Other	Lipopolysaccharide	Low biological activity
	Lewis X/Y blood group homology	Autoimmunity

H. pylori induces IL-8 production [Crabtree *et al.* 1995] and activates nuclear factor-kappa B (NF-κB) in gastric epithelial cells both *in vivo* and *in vitro* [Keates *et al.* 1997; van Den Brink *et al.* 2000]. NF-κB regulates a variety of genes whose products are involved in cell growth, inflammation and immune responses [Siebenlist *et al.* 1994].

The different patterns of cytokines produced during the gastric immune response to *H. pylori* affect the outcome of the infection. T helper 1 (Th1) cells mediate cell-mediated immunity, strong macrophage activation, cytotoxicity and help for B cell production of opsonizing and complement-fixing antibodies; in contrast, Th2 cells induce the production of high levels of antibodies of all isotypes, including IgE, tend to inactivate macrophages, recruit and activate eosinophils and mast cells (Fig. 3) [Del Prete 1998]. *H. pylori* infected gastric mucosa T cells display a predominant Th1 phenotype with IFN-γ, TNF-α and IL-12 expressed [D'Elios *et al.* 1997; Bamford *et al.* 1998]. Th2 cytokines, particularly IL-4 and IL-10, are important

in balancing and quenching some immunopathological effects of a polarized Th1 response [Smythies *et al.* 2000; Haeberle *et al.* 1997]. Enteric helminthic parasites driving a polarized Th2 response seem to attenuate *Helicobacter* induced gastric atrophy in mice [Fox *et al.* 2000]. Therefore a combination of a Th1 and Th2 response appears to probably be the best for a future protective immunization.

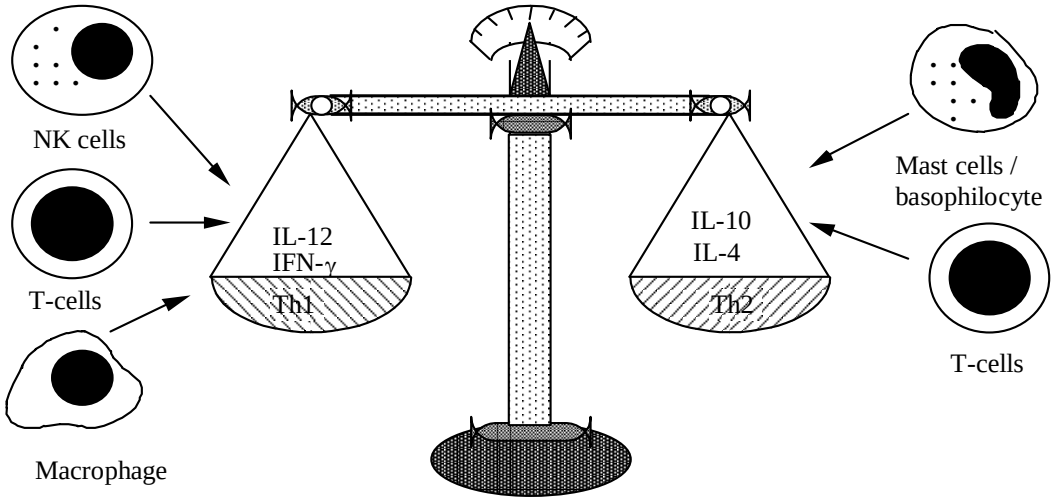


Fig. 3 The Th1/Th2 balance in infection [Kemp *et al.* 1996].

Free radical injury and *H. pylori*

Free radicals and lipid peroxidation

Free radicals are atoms or molecules that possess one unpaired electron on their outer orbital. They are very unstable and quite reactive and are produced in both normal and pathological processes. Reactive oxygen species (ROS) are generated from endogenous and exogenous sources such as diet, cigarette smoke, radiation and air pollution. Excess of ROS are thought to mediate a large portion of the tissue damage produced upon inflammation, ischemia and reperfusion and may also be involved in the pathogenesis of many diseases including heart disease, inflammatory and neurodegenerative disease and carcinogenesis [McCord 2000; Berdanier *et al.* 2001; Kong *et al.* 2000; Emerit *et al.* 2000; Cavalca *et al.* 2001].

Free radical reactions tend to proceed as chain reactions and not terminated until the free radical is deactivated by a chain reaction breaking antioxidant [Gutteridge *et al.* 2000]. Free radicals such as superoxide anion radical ($O_2^{\bullet}$) and

hydroxyl radical ($OH^{\bullet}$) can react with each other and form new free radicals and it can react with a non-radical, resulting in a chain reaction which can lead to the formation of a plethora of other radicals with fatty acids which initiate lipid peroxidation. ROS are generated in the inflammatory response with DNA and consequent genetic damage and may also promote carcinogenesis by inducing proto-oncogen expression, by causing generation of genotoxic products or by converting procarcinogens [Wei 1992; Wang D *et al.* 1998; Tahara 1998]. The life span of free radicals are very short, counted as μs, which are difficult to be measured. Most common used research model is lipid peroxidation and their degraded product lipid peroxides [Babbs *et al.* 1990].

H. pylori and free radical damage

Epidemiological studies suggested that gastric cancer and precancerous lesions may be caused by ROS and that dietary antioxidants (eg. β-carotene, α-tocopherol, vitamin C and retinol) can prevent stomach cancer [Buiatti *et al.* 1990; Stahelin *et al.* 1991; Drake *et al.* 1996; Aldoori *et al.* 1997]. Evidence is rapidly accumulating that *H. pylori* is a major risk factor for human gastric adenocarcinomas [Tsugane *et al.* 1994; Hansson *et al.* 1996; Watanabe *et al.* 1998; Hahm *et al.* 1998]. *H. pylori* infection has been associated with lower concentrations of vitamin C, α-tocopherol and β-carotene in the gastric juice and/or gastric mucosa [Waring *et al.* 1996; Correa *et al.* 1998; Zhang *et al.* 1998; Jarosz *et al.* 2000; Zhang *et al.* 2000].

H. pylori enhances production of ROS in gastric cells and membrane damage and antioxidants can inhibit this increasing [Bagchi *et al.* 1996]. Increased production of ROS in the gastric mucosa was correlated to *H. pylori* density [Zhang Q *et al.* 1997]. A high oxidative stress and high level of ROS was found in the gastric mucosa of *H. pylori* infected individuals and gastric antioxidative capacity was increased after the eradication of *H. pylori* [Khaled *et al.* 1998]. *H. pylori* produces a neutrophil-activating protein [Evans *et al.* 2000] and induces expression of IL-8 and intercellular adhesion molecules in gastric epithelial cells [Testerman *et al.* 2001; Crowe *et al.* 1995], which can also activate infiltrating leukocytes to release ROS. Cross-talk between nitric oxide (NO), superoxide and molecular oxygen yield a variety of NO intermediates, some of which are potent nitrosating agents that will produce mutagenic and carcinogenic nitrosamines [Inoue *et al.* 1999; Grisham *et al.* 2000]. *H. pylori* directly decreases cellular glutathione (GSH) concentration in gastric epithelial

cells *in vitro* [Beil *et al.* 2000] and increase the level of lipid peroxidation and activated GSH turnover in a mongolian gerbil model [Suzuki *et al.* 1999].

Antioxidants and their function

Antioxidants are naturally occurring or synthetic substances that inhibit or retard the oxidation of other molecules by preventing the formation of radicals, scavenging them, or by promoting their decomposition [Gutteridge *et al.* 2000; Young *et al.* 2001]. In general, synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitution, whereas natural antioxidants can be phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), or carotenoids as well as ascorbic acid [Velioglu *et al.* 1998]. Antioxidants are classified by function into four categories (Table 3). Radical scavenging antioxidants have the greatest advantage with their chemical structure and a novel function by which they regulate gene expression of the cell. [Noguchi *et al.* 2000]. Antioxidants inhibit *H. pylori* induced tissue damage by inhibiting production of ROS from neutrophils and scavenging ROS (Fig. 4).

Many food constituents showing antioxidant activity have been found inhibitory effect on *H. pylori* growth *in vitro* and some of them also showed inhibitory effect in animal models (Table 4).

Table 3 Antioxidant defenses against free radicals modified from [Niki *et al.* 1995].

Antioxidants	Description	Active agents
Preventive antioxidants	Suppress the formation of free radicals	Catalase, glutathione peroxidase, glutathione S-transferase, transferrin, lactoferrin, superoxide dismutase, carotenoids, vitamin E
Radical-scavenging antioxidants	Scavenge radicals to inhibit chain initiation and break chain propagation	*Hydrophilic:* vitamin C, uric acid, bilirubin, albumin *Lipophilic:* vitamin E, carotenoids, flavonoids, ubiquinol
Repair and de novo enzymes	Repair the damage and reconstitute membranes	Lipase, protease, DNA repair enzymes, transferase
Adaptation	Generate appropriate antioxidant enzymes and transfer them to the right site at the right time and in the right concentration	

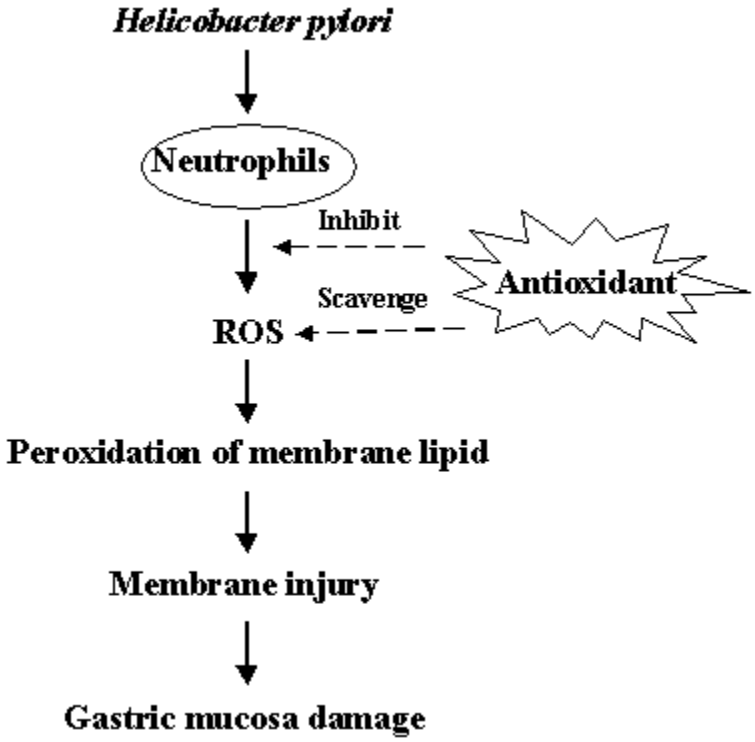


Fig. 4 The possible mechanism of antioxidants against *H. pylori* induced damage.

Vitamin C

Vitamin C (ascorbic acid) is a water-soluble antioxidant required for many biological functions [Levine 1986; Rebouche 1991] and it scavenges ROS to provide protection against oxidative DNA damage at a normal physiologic concentration [Drake *et al.* 1996; Noroozi *et al.* 1998]. Vitamin C is actively secreted into the gastric lumen and the concentrations in gastric juice and mucosa may be particularly important for effectively converting nitrites and nitrite derived mutagens [Cohen *et al.* 1995; Drake *et al.* 1996; Waring *et al.* 1996]. There is strong and complex interaction between chronic *H. pylori* infection, chronic deficiency and/or defective intragastric secretion of vitamin C and distal gastric carcinogenesis of the intestinal type [Zhang *et al.* 1998; Reed 1999]. Vitamin C inhibit *H. pylori* both *in vitro* and *in vivo* [Zhang HM *et al.* 1997; Wang X *et al.* 1998a; Paper V]. The inhibitory effect appears to be specific for *H. pylori* and *Campylobacter* species, as various *E. coli* strains, *Salmonella*, and *Vibrio* species were unaffected *in vitro* [Zhang HM *et al.* 1997].

Table 4 Inhibition of *H. pylori* by food antioxidants.

Food antioxidants	MIC[a] (μg/ml)	Inhibition in	Reference
Vitamin C	128-2,048	Gerbils	[Zhang HM *et al.*
	500-2,000	Mice	1997]
			[Paper V]
Algal meal	312.5-2,500	Mice	[Paper V]
(astaxanthin)	(6.25-50)		
Lycopene	100-400	Mice	[Yaquian 2001]
Tea catechins	2-1,024	Gerbils	[Mabe *et al.* 1999]
	17.8-167		[Yee *et al.* 2000]
Flavonoids	10-20	–	[Bae *et al.* 1999]
Garlic extract		–	
• Aqueous	2000-5000		[Cellini *et al.* 1996]
• Oil	10-100		[Ohta *et al.* 1999]
• Powder	250-500		[O'Gara *et al.* 2000]
Fish oils			
• Icosapentaenoic acid docosahexaenoic acid	+	–	[Drago *et al.* 1999] [Thompson *et al.* 1994]
	0.18-0.5 mM		
• ϖ-3-fatty acid			
Chili (Capsaicin)	>10	–	[Jones *et al.* 1997]
Red wine	12.5-50	Mice	[Mahady *et al.* 2000; Daroch *et al.* 2001; Mayo *et al.* 2000]
Rose oil	100-2000	–	[Boyanova *et al.* 1999]

[a]: MIC=minimal inhibitory concentrations; +: inhibition effect; –: not tested *in vivo*.

Gerbils or mice orally treated with 10 mg of vitamin C daily following *H. pylori* infection showed a significant lower gastric colonization than control animals [Zhang HM *et al.* 1997; Paper V]. Patients with proven chronic gastritis and *H. pylori* infection received a high dose of vitamin C (5g per day) daily for 4 weeks resulted in a *H. pylori* eradication of 30% [Jarosz *et al.* 1998]. In those patients there was also a highly significant rise in total vitamin C concentration of the gastric juice persisted for at least 4 weeks post-treatment.

Carotenoids

More than 600 carotenoids have been identified in nature [Shahidi *et al.* 1998]. Approximately 50 carotenoids have been found in the human diet and 20 have been identified in plasma and body tissues [Faure *et al.* 1999]. β-carotene is one of the most common carotenoids and is the primary precursor to vitamin A. It can strengthen

the immune system and provide protection against cancer [Chew 1993; Stahelin *et al.* 1991].

Astaxanthin (3,3'-dihydroxy-*β,β*-carotene-4,4'dione) is a carotenoid found in many different organisms in nature but the main dietary sources to humans are found in crustaceans and other seafood, especially salmons. It has attracted considerable interest in recent years because of its superior antioxidative activity to most other hydrophobic antioxidants and possible role in delaying or preventing degenerative diseases, and also a source of pigment in aquaculture feeds [Fan *et al.* 1998; Jyonouchi *et al.* 1995; Jyonouchi *et al.* 1996; Kurashige *et al.* 1990; Okai *et al.* 1996; Palozza *et al.* 1992; Pettersson *et al.* 1999; Tanaka *et al.* 1995; Todd Lorenz *et al.* 2000]. The astaxanthin has a significantly greater antioxidant effect than related carotenoids such as canthaxanthin and β-carotene and vitamin E (Fig. 5) [Miki 1991; Naguib 2000], is most effective to stimulate immune defenses [Jyonouchi *et al.* 1996; Okai *et al.* 1996] and has higher anti-tumor activity than canthaxanthin and β-carotene [Chew *et al.* 1999]. An astaxanthin-rich algal meal inhibit *H. pylori in vitro* and *in vivo* and decreased lipid peroxidation induced by *H. pylori* infection [Paper V]. Astaxanthin suppresses interferon-γ (IFN-γ) *in vitro* [Jyonouchi *et al.* 1996] and increases IL-4 production *in vivo*, suggesting that astaxanthin promotes a shift from a predominantly Th1 response to a mixed Th1/Th2 response [Paper VI]. Astaxanthin was found to inactivate NF-κB in a vitro model with HeLa cells [Pettersson S, personal communication]. A first human trial on astaxanthin-rich algal meal was done in Australia and showed that astaxanthin improved dyspeptic symptom of gastritis patients and ameliorated *H. pylori* infection [Lignell *et al.* 1999]. Thus, astaxanthin may protect the mucosal surface both by decreasing phagocyte activation and via its intrinsic antioxidant activity similar to rebamipide [Danielsson *et al.* 1998; Iinuma *et al.* 1998].

Vitamin E (Tocopherols)

Vitamin E is an essential lipid-soluble vitamin in the human diet, and is found in cell membranes and plasma lipoproteins. Alpha-tocopherol is one of the most important free radical scavengers and inhibits lipid peroxidation in cell membranes by peroxyl radicals [Evans 2000]. Epidemiological studies showed a low serum level of alpha-tocopherol in patients with gastric dysplasia [Correa *et al.* 1998]. Gastric alpha-

tocopherol concentration is affected by *H. pylori* associated gastric histological changes [Zhang *et al.* 2000]. However, a 5-year clinical trial involving male smokers with atrophic gastritis failed to show any protective effect of vitamin E supplement [Varis *et al.* 1998].

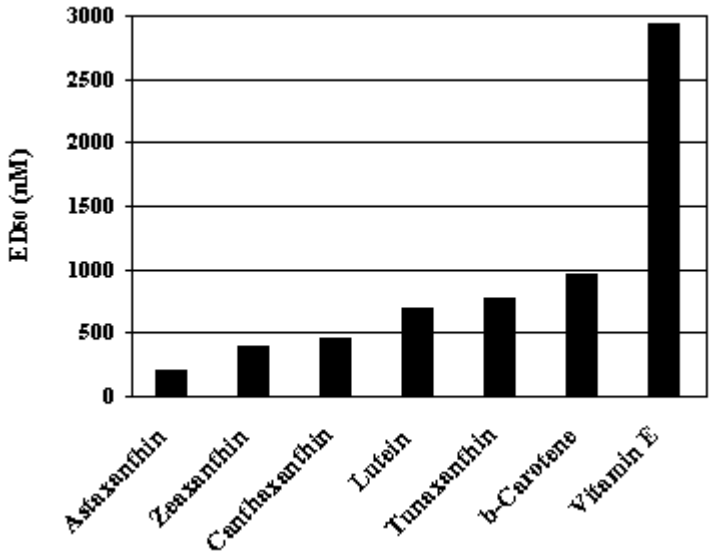


Fig. 5 Minimal concentration of 50% inhibition of lipidperoxidation (ED_{50}) in carotenoids and vitamin E [Miki 1991].

Helicobacter pylori infection and gastric diseases

Non-ulcer dyspepsia (NUD)

Dyspepsia is not a clinical diagnosis but consist of a variable combination of symptoms including abdominal pain or discomfort, postprandial fullness, abdominal bloating, early satiety, nausea, vomiting, heartburn and acid regurgitation. It can be early symptom of underlying illness like peptic ulcer disease or even gastric carcinoma, but often no lesion can be found [Moayyedi *et al.* 2000].

H. pylori colonizes the gastric antrum in one third to one half of patients with NUD [Jaakkimainen *et al.* 1999]. Gastroduodenal dysfunction may be associated with *H. pylori* infection, but there is little evidence for a causal link with dyspepsia [Quina 1998] and insufficient evidence to confirm or refute the existence of a modest association [Danesh *et al.* 2000a]. There are conflicting results based on clinical trials [Quina 1998; Jaakkimainen *et al.* 1999; Xia *et al.* 1999; Danesh *et al.* 2000b]. More recently Moayyedi *et al* suggested that *H. pylori* eradication might be cost effective

treatment for NUD in infected patients but further evidence is still needed [Moayyedi *et al.* 2000].

Gastritis

Warren and Marshall first identified spiral organisms which closely applied to the gastric epithelium in active chronic gastritis [Warren *et al.* 1983], today *H. pylori* is accepted as the cause of the most common form of chronic gastritis. It is widely appreciated that chronic gastritis is a common denominator linking peptic ulceration, gastric carcinoma, and lymphoma and with a histological picture of chronic inflammation, atrophy and intestinal metaplasia. Other environmental and dietary factors including stress, excessive alcohol consumption, bile reflux, and high intake of salted food are also involved in this pathological process.

The Sydney System for the classification of gastritis emphasized the importance of combining topographical, morphological, and etiological information into a schema that would help to generate reproducible and clinically useful diagnoses [Misiewicz 1991]. The discovery of *H. pylori* has totally changed the classification and grading of gastritis and the Sydney System had been modified by a group of gastrointestinal pathologists (Table 5).

Peptic ulcer disease

The close relationship between acid-induced gastric metaplasia, *H. pylori* and active chronic duodenitis was first emphasized by Wyatt et al [Wyatt *et al.* 1987]. A hypothesis of factors influencing the development of duodenal ulcer versus gastric ulcer/cancer is proposed. Different patterns of *H. pylori* gastritis are associated with profound alterations in acid output. In antral predominant gastritis, acid production from the largely dominant unaffected corpus is enhanced whereas corpus inflammation is associated with hypochlorhydria (Fig. 6). These changes have an important bearing on peptic ulcer pathogenesis.

Gastric cancer

Infection with *H. pylori* has been classified as exposure to a Group I carcinogen by the International Agency for Research on Cancer (IARC) in 1994 [Moller *et al.* 1995]. The most important epidemiological evidence supporting this association was provided by prospective investigations which showed that infected

individuals has a four-fold or higher increased risk of subsequently developing gastric cancer. This has later been confirmed and it now appears that, after allowing for the effects of atrophy and aging, the relative risk may be in the order of nine- or ten-fold [Parsonnet *et al.* 1991; Nomura *et al.* 1991; Talley *et al.* 1991]. A recent 4.5-year follow-up study in Linqu of China with one of the worlds highest rates of gastric

Table 5 Classification of chronic gastritis in man [Dixon *et al.* 1996].

Type of gastritis	Etiologic factors	Gastritis synonyms
Nonatrophic	*H. pylori*	Superficial
	Other factors	Diffuse antral gastritis (DAG)
		Chronic antral gastritis (CAG)
		Interstitial-follicular
		Type B
Atrophic		
Autoimmune	Autoimmunity	Type A
Multifocal atrophic	*H. pylori*	Type B, Type AB
	Dietary	Environmental
	Environmental factors	Metaplastic
Special forms		
Chemical	Chemical irritation	Type C
Radiation	Radiation injury	
Lymphocytic	Idiopathic	Celiac disease-associated
	H. pylori	
Noninfectious		
Eosinophilic	Food allergies	Allergic
Other infectious	Bacteria (other than *H. pylori*), Virus, Fungi, Parasites	Phlegmonous

cancer showed the presence of *H. pylori* was associated with an increased risk of progression to dysplasia or gastric cancer, and the risk of progression was decreased among the subjects with high vitamin C intake and serum levels [You *et al.* 2000].

Helicobacter pylori inducing direct DNA damage [Schmausser *et al.* 2000] and inflammation, cell apoptosis and proliferation [Peek *et al.* 2000; Xia *et al.* 2001] suggest a link between chronic *H. pylori* infection and development of adenocarcinoma of the stomach (Fig. 7). There is also evidence that *H. pylori* induce intestinal type gastric adenocarcinoma in 40% of infected Mongolian gerbils after 62 to 78 weeks inoculation [Watanabe *et al.* 1998; Ikeno *et al.* 1999]. *H. pylori* infection

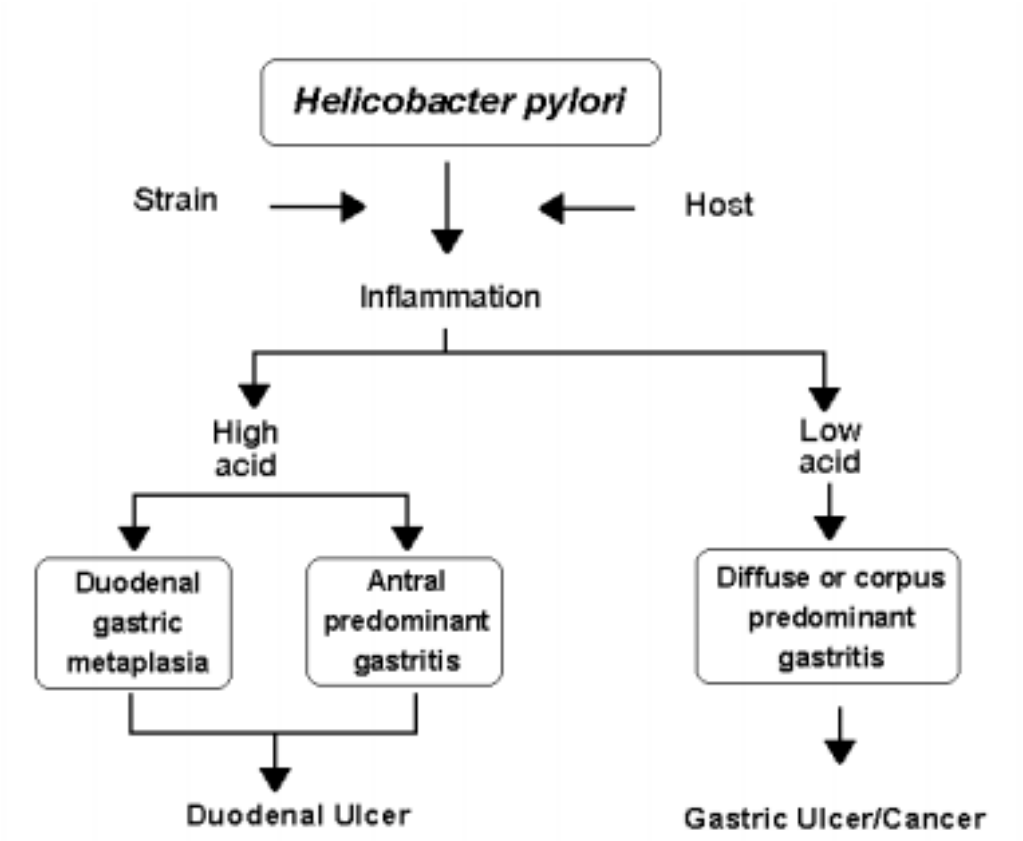


Fig. 6 A hypothesis of the development of peptic ulcer/gastric cancer [Kuipers 1999].

enhances glandular stomach carcinogenesis in Mongolian gerbils or mice treated with a chemical carcinogen like MNU, MNNG or sodium nitrite [Tatematsu *et al.* 1998; Sugiyama *et al.* 1998; Wang X *et al.* 1998b; Shimizu *et al.* 1998]. A multistep model for the development of gastric cancer has been proposed from superficial gastritis progressing to chronic atrophic gastritis, intestinal metaplasia, dysplasia and ultimately carcinoma [Correa 1992b]. Besides *H. pylori*, environmental factors usually implicated in the development of gastric cancer is diet; specially dietary salt and nitrate promote cancer development while antioxidants (ascorbic acid, β-carotene, vitamin E) can inhibit the development (Fig. 7).

Gastric lymphoma

An accumulation of lymphoid tissue in the stomach is a precursor state for development of lymphoepithelial lesions (LEL) of the gastric mucosa, low and high grade MALT (mucosa-associated lymphoid tissue) lymphoma. These lesions are strongly associated with the presence of *H. pylori* infection [Isaacson 1999; Bouzourene *et al.* 1999; Morgner *et al.* 2000a; Morgner *et al.* 2001].

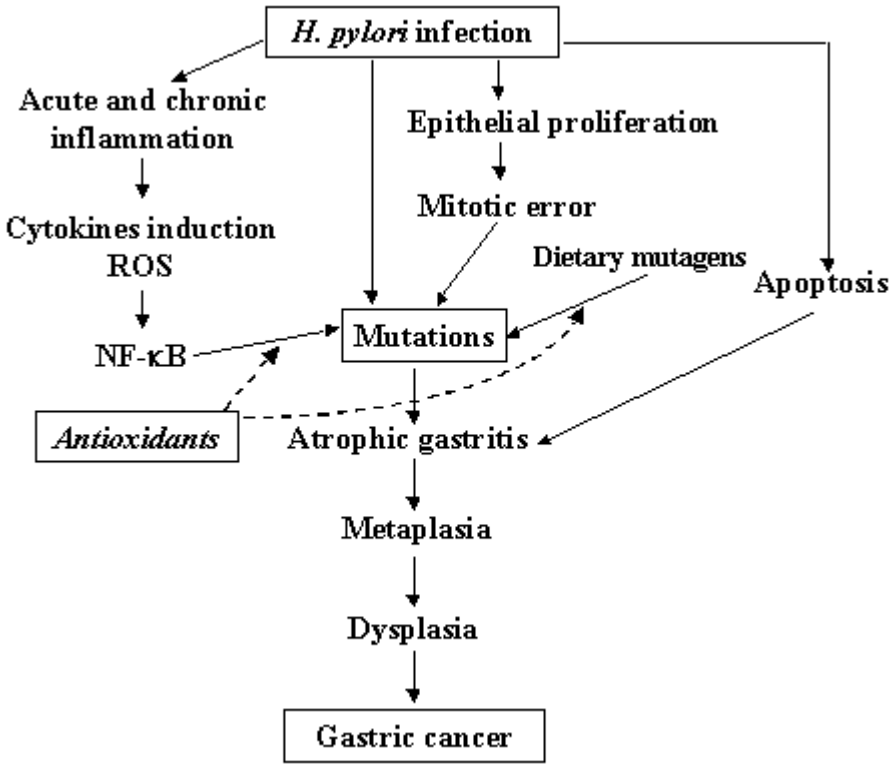


Fig. 7 A model for gastric carcinogenesis incorporating *H. pylori* infection modified from [Parsonnet 1993; Kuipers 1999]. Dotted arrows indicate protective factor.

The MALT lymphoma formation and growth is probably antigen driven and it has been suggested that *H. pylori* could serve as such and proliferation of B cells could be dependent on cognate help from *H. pylori* specific T-cells [Hussell *et al.* 1996; Isaacson 1999]. B and T cells are recruited to the gastric mucosa as part of the complex immune response to *H. pylori*. Conversion to high-grade lymphoma might not require the presence of *H. pylori* [Enno *et al.* 1998; Bouzourene *et al.* 1999; Isaacson 1999]. The histopathological features of low grade primary gastric lymphoma recapitulate structures of Peyer's patches [Isaacson 1996; Hsi *et al.* 1998]. Transformation of low grade MALT-lymphoma to high grade primary gastric lymphoma is well recognized and often a mixture of both can be found [De Wolf-Peeters *et al.* 1999; Chan *et al.* 1990; Matolcsy *et al.* 1999]. Sometimes only a high grade primary gastric lymphoma is found and a de novo lymphoma can not be ruled out [Bouzourene *et al.* 1999].

Antibiotic treatment can be successful in an earlier phase of low-grade MALT-lymphoma [Wotherspoon *et al.* 1993; Raderer *et al.* 2000]. More recently Morgner reported that high-grade gastric lymphoma in 8 patients completely regressed after *H. pylori* eradication [Morgner *et al.* 2001]. The mechanisms behind the decrease in *H. pylori* lymphoma formation are uncertain. Mucosa atrophy, intestinal metaplasia and reduction of tissue neutrophils have been suggested.

Although *H. pylori* may be the most common cause of many gastrointestinal MALT lymphoma, it is not the only causative organism. *H. heilmannii* [Morgner *et al.* 2000b], other non-*H. pylori* bacteria [Jonkers *et al.* 1997] and some protozoa [Otrakji *et al.* 1990] have been observed in gastric lymphomas specific to involved regions. Animal experiments have revealed lymphoma induction, such as life-long infection of the stomach of Balb/c mice with *H. felis* resulting in gastric MALT lymphoma [Enno *et al.* 1995; Enno *et al.* 1998]. High grade lymphoma was found in *H. pylori* infected C57BL/6 mice after 13 months [Paper IV]. There were both gastric lymphoid tissue and glandular hyperplastic lesions found in Swiss mice with 13 months *H. felis* infection [Ferrero *et al.* 2000].

Treatment, antibiotic resistance and vaccine development

Triple therapy that includes a proton pump inhibitor (PPI) or ranitidine bismuth citrate (RBC) in combination with two antibiotics has been accepted as the standard of care for the treatment of *H. pylori* infection [Van Oijen *et al.* 2000; Nakajima *et al.* 2000]. The recommended antibiotics include amoxicillin, clarithromycin, and metronidazole [Van Oijen *et al.* 2000]. Although these drug combinations have proven to be curative, they are complicated, expensive and not always effective since *H. pylori* strains are becoming resistant to antibiotics [Leung *et al.* 2000; Lahaie *et al.* 2000]. Eradication therapy in asymptomatic patients or in those with undefined dyspepsia might cause antibiotic resistance of *H. pylori* and other pathogenic bacteria present in the gastrointestinal tract and exacerbate gastro-esophageal reflux disease [McCarthy 1998; Stanghellini *et al.* 2000].

The so-called mucosal defensive agents including several drugs such as rebamipide [Nebiki *et al.* 1998], ecabet sodium [Ohkusa *et al.* 1998], polaprezinc [Kashimura *et al.* 1999], plaunotol [Fukuda *et al.* 1995], sofalcone [Sakaki *et al.* 1998] and sucralfate [Adachi *et al.* 2000] were used as anti-ulcer drugs. A recent

review demonstrated that such mucosal defensive agents improve the cure rate when used with existing dual therapy regimens for eradicating *H. pylori* infection, but not with triple therapy [Hojo *et al.* 2000]. The generally assumed mechanism of action of these agents involves up-regulation of gastric mucosal defenses while the process of recovery from mucosal injury occurs. Some of these drugs have also been shown to have anti-*H. pylori* activities [Hayashi *et al.* 1998; Shibata *et al.* 1995; Koga *et al.* 1996; Slomiany *et al.* 1995]. Their effectiveness in eradicating *H. pylori* is not well established. Rebamipide, a novel ulcer-healing compound with antioxidative and anti-inflammatory properties, protects gastric mucosal injury by scavenging hydroxyl radical and inhibiting neutrophil activation or lipid peroxidation [Iinuma *et al.* 1998; Hahm *et al.* 1998; Kim *et al.* 2000].

Two anti-adhesive therapies on *H. pylori* infection have been investigated in animal models [Mysore *et al.* 1999; Wang *et al.* 2001]. The use of 3'-sialyllactose sodium salt (3'SL), an oligosaccharide that occurs naturally in human and bovine milk and that can inhibit the adhesion of *H. pylori* to human epithelial cells *in vitro*, was achieved with a 35% cure rates in rhesus monkeys [Mysore *et al.* 1999]. Bovine milk glycoconjugates were tested in our mouse model and showed potencies to inhibit *H. pylori* infection with 10-30% healing rate [Wang *et al.* 2001]. Therefore, these compounds could be considered as candidates for non-antibiotic strategies against *H. pylori* infection in man. However, the results of orally administrated hyperimmune bovine colostral immune globulins, 3'-sialyllactose or recombinant human lactoferrin in healthy *H. pylori* infected volunteers were disappointing [Opekun *et al.* 1999]. Higher doses, longer duration of therapy, adjunctive acid suppression, or a combination could yield better results.

The feasibility of producing an effective vaccine against *H. pylori* has been questioned due to the fact that natural immunity appears to be inadequate for clearing the infection. Therapeutic intragastric vaccination against *H. pylori* in mice eradicates an chronic infection and confers protection against reinfection [Ghiara *et al.* 1997]. The antigenic potential of urease has been demonstrated clearly in animal studies and prototype vaccine studies in mice and monkeys have proved encouraging [Ermak *et al.* 1998; Dubois *et al.* 1998; Solnick *et al.* 2000; Lee 2001]. However, it is likely that *H. pylori* urease or its individual subunits in combination with other defined *H. pylori* antigens may prove a more effective vaccine against infection by this bacterium. In a

series of studies, mice immunized with both heat shock protein A (HspA) and urease B subunit (UreB) or the engineered heat shock protein (GroES) and UreB, as well as vacuolating cytotoxin (VacA) and urease were found to be highly protecting from infection [Marchetti *et al.* 1995; Ferrero *et al.* 1995; Hocking *et al.* 1999]. DNA vaccines encoding *H. pylori*-Hsp induce a significant immune response against *H. pylori* and decrease gastric mucosal inflammation in mice, indicating that a DNA vaccine can be a new approach against *H. pylori* in humans [Todoroki *et al.* 2000].

Animal models

A wide range of animal models have been developed to investigate the pathogenesis of *Helicobacter* infection and therapy for the disease over the last decade. Each of these animal models has distinct advantages and disadvantages (Table 6).

Mouse models

Although there is no single model that is the best for all applications, on the basis of cost and availability of immunological reagents and genome information, the mouse is undeniably the most convenient and appropriate today.

H. felis

In 1990, Lee and colleagues reported that germ-free Swiss Webster mice could be infected with *H. felis*, showing an active/chronic gastritis similar to the human pattern of inflammation [Lee *et al.* 1990]. It was subsequently shown that *H. felis* could also infect conventional mice and that the severity of disease varied greatly with the inbred strain of mouse used [Mohammadi *et al.* 1996]. Long-term (>22 months) *H. felis* infection in Balb/c mice developed gastric MALT lymphoma lesions [Enno *et al.* 1995]. In spite of the fact that *H. felis* infection yield more severe disease than *H. pylori* infection of mice does, *H. felis* apparently does not contain the *cag* pathogenicity island, nor does it adhere tightly to gastric epithelial cells. Since both of these traits are accepted virulence factors for *H. pylori*, the ultimate usefulness of the *H. felis* mouse model to study *H. pylori* pathogenesis may be questionable.

H. pylori

In the late 1980s and early 1990s, many laboratories unsuccessfully attempted to establish mouse models of *H. pylori* infection [Cantorna *et al.* 1990]. In 1991 Japanese researchers reported that *H. pylori* could infect nude mice continuously and euthymic mice temporarily [Karita *et al.* 1991]. This result encouraged other

Table 6 Animal models of *Helicobacter* infection [Lee 1999].

Animals	Colonized by	Advantage	Disadvantage
Primate	*H. pylori*	Closest animal species to human, endoscopy possible, gastric physiology similar to human	Expensive, colonized by endemic strains, presence of *H. heilmannii*-like (HHLO) bacteria
Cat and dog	*H. pylori* *H. felis*	Gastric physiology similar to human	Gnotobiotic and SPF animals expensive, HHLO present in normal cats
Ferret	*H. mustelae*	Natural infection, useful for vaccine studies, gastric physiology similar to human	Pattern of colonization varies from human, predominantly chronic gastritis only
Guinea pig	*H. pylori*	IL-8 homologue, active chronic gastritis	Little used
Mouse	*H. pylori* *H. felis* *H. heilmannii*	Economical good for testing vaccines/antimicrobials	Does not mimic human pathology
Mongolian gerbil	*H. pylori*	Chronic/active antral gastritis, gastric ulcers, adenocarcinoma induced with infection alone	Lack of immunological reagents and transgenic/knockout strains
Gnotobiotic piglet	*H. pylori*	Colonization pattern similar to human, gastric physiology similar to human, ulcers observed	Chronic gastritis only, expensive short-term experiments only

researchers to investigate *H. pylori* infection in immunocompetent mice and several groups succeeded independently [Marchetti *et al.* 1995; Aleljung *et al.* 1996; Paper I; Lee *et al.* 1997]. Using fresh clinical *H. pylori* isolates can infect mice transiently [Marchetti *et al.* 1995] and a mouse-adapted *H. pylori* Sydney strain was established with long-term and high bacterial colonization in mice [Lee *et al.* 1997]. Gastrointestinal colonization of *H. pylori* in BALB/cA mice was monitored by

heparin magnetic separation [Aleljung *et al.* 1996] and the infection was followed by both spiral and coccoid form of *H. pylori* in BALB/cA mice till 30 weeks [Paper I]. The histopathological evidence persisted during 30 weeks and *H. pylori* specific antibodies were detected after 4 to 30 weeks by ELISA and immunoblot.

The choice of *H. pylori* strain is important for establishing the infection in a mouse model. Various *H. pylori* strains gave different results in different strains of mice. The capacity of *H. pylori* strains to colonize different strains of mice is both bacterial strain and host dependent [Danon *et al.* 1998b; van Doorn *et al.* 1999; Ferrero *et al.* 1998]. Inoculation by a mixture of 9 *H. pylori* strains in 4 different mouse strains were detected mostly one strain (119/95) by RAPD-PCR [Paper III]. *H. pylori* bacteria were shown to interact intimately with the gastric mucosa of the infected mice. The value of optimizing the diet to allow a maximal colonization and inflammation score for clinical isolates as well as mouse-adapted strains is very important. One of 4 tested commercial diets showed the best result in sustain *H. pylori* infection [Paper II]. This diet is without fish product and heated to 140-150^{o}C [Wang X *et al.* 2000]. Long-term infection of *H. pylori* in C57BL/6 mice induced a high-grade lymphoma [Paper IV].

Animal adapted *H. pylori* strains did not exhibit differences in the LPS-associated Lewis antigen phenotype [Wirth *et al.* 1998] or in the production of major cellular proteins [Ferrero *et al.* 2001b]. In addition, the use of RAPD analysis, a very sensitive fingerprinting technique for detecting genetic rearrangements or point mutations in bacteria, did not reveal any major modifications in DNA arrangement of isolates following multiple passages *in vitro* or *in vivo* [Wirth *et al.* 1998; Paper III].

Vaccine candidates have been studied in *H. pylori* infection mouse models [Ghiara *et al.* 1997; Crabtree 1998; Gomez-Duarte *et al.* 1999; Ruiz-Bustos *et al.* 2000]. New therapeutic strategies against *H. pylori* have been investigated in the mouse model [Paper VI; Paper V; Wada *et al.* 1999; Wang *et al.* 2001].

H. heilmannii

This organism is known to infect the human stomach, probably acquired from domestic pets, and was first called *Gastrospirillum hominis* [McNulty *et al.* 1989]. Genetic studies on infected gastric tissue indicated that these bacteria also belonged to the genus *Helicobacter*, named *Helicobacter heilmannii* [Solnick *et al.* 1993]. *H.*

heilmannii was firstly isolated in pure culture in 1996 [Andersen *et al.* 1996]. There have been few studies on *H. heilmannii* infection in mice [Eaton *et al.* 1993; Lee *et al.* 1993; Danon *et al.* 1998a; Peterson *et al.* 2001]. Gastric colonization with these bacteria is quite remarkable in both natural hosts and rodents. Removal of these bacteria by preventive or therapeutic with immunization would indeed be a substantial achievement. Urease-based mucosal immunization protects Balb/c mice against *H. heilmannii* infection and significantly reduces a preexisting infection but immunization itself aggravates gastric atrophy [Dieterich *et al.* 1999]. *H. heilmannii* infected mice might be a good screen for new mucosal adjuvants.

Mongolian gerbil model

Several recent reports showed that mongolian gerbils can be infected with *H. pylori* and develop gastritis, gastric ulcers, intestinal metaplasia and gastric cancer [Matsumoto *et al.* 1997; Honda *et al.* 1998; Watanabe *et al.* 1998; Ikeno *et al.* 1999]. *H. pylori* has shown both co-initiating and promoting effects on chemical-induced (MNU or MNNG) gastric carcinogenesis in this model [Tatematsu *et al.* 1998; Sugiyama *et al.* 1998]. Most recently there is a host response study in this model showed that the frequency of seropositivity increased over time and gerbils developed gastric ulcer after 26-week infection showed significantly higher serum IgG level than did gerbils who developed hyperplasia [Kumagai *et al.* 2001]. The mongolian gerbils may represent a useful model to improve our understanding of the pathogenesis of *H. pylori* related human gastric diseases.

Guinea pig model

Guinea pigs, like humans, have a dietary requirement for vitamin C and secret a well-characterized homologue of interleukin-8 (IL-8) [Yoshimura *et al.* 1993]. The guinea pig model for *H. pylori* infection was described in several studies [Shomer *et al.* 1998; Sturegard *et al.* 1998]. Mild to moderate multifocal antral gastritis was reported in these animals infected by the mouse adapted *H. pylori* Sydney strain. This model is useful to study the interaction of *Helicobacter* and low level of vitamin C in gastric carcinogenesis.

Ferret model

Virtually all commercial colonies of ferrets in America and many colonies elsewhere in the world are naturally infected with *H. mustelae*. This gastric *Helicobacter* has been associated with chronic gastritis and peptic ulcer as well as MALT lymphoma in these animals [Fox *et al.* 1988; Fox *et al.* 1990; Erdman *et al.* 1997]. Ferrets have been proposed as a suitable model to study the pathogenesis and epidemiology of *Helicobacter* associated chronic gastritis and gastric cancer [Fox *et al.* 1990; Fox *et al.* 1993; Yu *et al.* 1995]. But as a bacterium, *H. mustelae* is different from *H. pylori*. It was shown that *H. pylori* will not colonize the *H. mustelae* free ferret [Fox *et al.* 1991b]. There are intercountry differences in ferret infection, and no inflammation was observed in 17 of 17 infected ferrets in the United Kingdom [Tompkins *et al.* 1988]. There are few reagents available for the use in immunological studies on this species.

Cat and dog model

Natural and experimental *H. pylori* infections of domestic cats were reported [Perkins *et al.* 1998; Esteves *et al.* 2000; Simpson *et al.* 2001]. Infected cats developed a lymphofollicular gastritis with small to moderate numbers of eosinophils and a moderate antral infiltration of neutrophils. Long-term natural *H. pylori* infection in cats offers a suitable model for study of *H. pylori* pathogenesis due to the physiology of immune cell reactivity and mucosa responses to a chronic injury [Esteves *et al.* 2000]. A beagle dog model had been reported for acute and chronic infection with *H. pylori* [Rossi *et al.* 1999]. An acute infection induces symptoms (vomiting and diarrhea) that disappear spontaneously and acute gastritis, with early recruitment of neutrophils, possibly mediated by *H. pylori*-induced IL-8, followed by the appearance of superficial erosion and of lymphoid follicles and chronic gastritis. Cat and dog models allow monitoring the consequences of *H. pylori* infection by gastroendoscopy. The disadvantage of these models is that the prevalence of gastric *Helicobacter spp.* in dogs and cats is high [Neiger *et al.* 2000; Strauss-Ayali *et al.* 2001].

Primate model

Primates are obvious candidates for investigation of *Helicobacter* infection. Rhesus monkeys are sometimes naturally colonized by *H. pylori* and suggested as a

model for spiral bacteria infection in humans [Dubois *et al.* 1994]. Experimental infection with *H. pylori* have been achieved by previous antibiotics treatment in several studies [Dubois *et al.* 1996; Mysore *et al.* 1999]. Evidence has also been obtained that vaccination induce an anti-*Helicobacter* activity in monkeys [Solnick *et al.* 2000; Lee 2001]. Studies in non-primates offer encouragement that experimental vaccination against *H. pylori* may be extended from mice to lager animals and humans. Besides the expensive costs for primate experiments, many colonies of primates are themselves infected with *H. pylori* that sometimes making it difficult to interpret the results.

Others

Gnotobiotic piglet model

Germ-free piglets were first used as an animal model for *Helicobacter* infection [Krakowka *et al.* 1987; Lambert *et al.* 1987]. This model has proved extremely useful in defining the importance of critical colonization factors, such as urease and cytotoxin-gene associated pathogenicity island [Eaton *et al.* 1996; Eaton *et al.* 2001]. Although barrier-born pigs were also infected with *H. pylori* and germ-free piglets can be conventionalized after infection, utilization of this model requires specialized facilities [Enroth *et al.* 2000]. In addition, while infected piglets have been followed for up to 120 days, they grow quite rapidly and it becomes impractical to maintain them much beyond 60 days. This model has not been widely used in vaccine studies, probably in part due to high cost and the need for these special facilities.

Rat model

In the first study in rats, *H. felis* induced a relatively mild lymphocytic gastritis after 2 to 8 weeks oral inoculation into germ-free rats [Fox *et al.* 1991a]. *H. pylori* caused little or no inflammation of the intact gastric mucosa in rats [Ross *et al.* 1992] and this model has not been much used for any great extent. Both VacA-/CagA- and VacA+/CagA+ strains of *H. pylori* induced only mild to moderate mucosal inflammation and no atrophic gastritis was observed even after one year [Li *et al.* 1998; Zeng *et al.* 1998; Li *et al.* 1999].

Transgenic mice model

Studies of transgenic and knockout mice specific strains will facilitate the understanding of *H. pylori* pathogenesis and the basis of successful prophylactic and therapeutic immunization. Gnotobiotic-transgenic mouse models can be developed to test hypotheses about the microbial and host factors that may determine the outcome of *H. pylori* infection in man [Falk *et al.* 2001]. The FVB/N transgenic mice engineered Lewis^{b} glycans and the FVB/N transgenic mice with ablation of parietal cells by expressing an attenuated diphtheria toxin A (*tox*176) were used to study *H. pylori* attachment-related pathology [Syder *et al.* 1999; Guruge *et al.* 1998]. In an insulin-gastrin transgenic mice strain, a synergistic interaction of hypergastrinemia and *H. felis* infection was found to cause parietal cell loss with progression to gastric cancer [Wang TC *et al.* 2000], whereas in gastrin-knockout mice *H. pylori* infection stimulated acid secretion, but parietal and ECL cells were unaffected [Chen *et al.* 2001]. It is likely that specific host genotypes affect predisposition to gastric diseases such as gastric cancer and that *H. pylori* signals to the host are influenced by gastrin.

Specific cytokine knockout mice strains have been used for studying host immune responses to *Helicobacter* infection and the role of a Th1 polarized response is confirmed to contribute the disease [Smythies *et al.* 2000]. For example IFN-γ seems essential in experimental *H. pylori* infection [Sawai *et al.* 1999] and IL-10 seems essential in experimental *H. felis* infection [Berg *et al.* 1998]. Results with IL-4 knockout and transgenic mice have not revealed marked differences against *H. pylori* infection, leaving the role of a Th2 response associated cytokines unclear [Chen *et al.* 1999]. Therapeutic immunization during a *H. pylori* infection stimulates an immune response, which reduces the infection via antibody independent mechanism by using transgenic and knockout mice [Sutton *et al.* 2000; Ernst *et al.* 2000]. More studies of transgenic and knockout mice specific strains will provide more vital information to understand the pathogenesis of *H. pylori* infection in the near future.

Aims of the present investigations

The broad aim of this study is to optimize a mouse model for *H. pylori* infection.

1. To study *H. pylori* infection by both spiral and coccoid forms of strain 17874, 25 and 553/93 in a mouse model with analysis of histopathological changes and systemic immune responses.
2. To compare how different rodent diets affect *H. pylori* infection in a BALB/cA mouse model and to develop a standardized diet in order to monitor the development of chronic type B gastritis.
3. To investigate which *H. pylori* strain(s) predominate in murine stomachs from inocula of nine freshly isolated strains and to compare the infection in 4 mouse strains to further optimize the mouse model.
4. To follow *H. pylori* infection with strain 119/95 for 13 months in C57BL/6 mice to elucidate if malignancy might develop and further study pathogenesis of *H. pylori* associated gastric diseases.
5. To explore whether antioxidants such as (i) astaxanthin from the microalgae *Haematococcus pluvialis* and (ii) vitamin C can suppress experimental *H. pylori* infection in the BALB/cA mouse model.
6. To investigate how an antioxidant astaxanthin-rich algal meal may modulate the T-lymphocyte response in BALB/cA mice which may affect *H. pylori* infection

Materials and Methods

Bacterial Strains:

Helicobacter pylori strains were grown on GAB-Camp agar (Gc Agar Base; Becton Dickinson, Lund, Sweden) supplemented with 10% horse serum [Soltez *et al.* 1988] and was incubated for 48 hours at 37^{o}C under microaerophillic conditions to obtain a maximal yield of spiral-shaped *H. pylori*. To obtain viable but non-culturable *H. pylori* cells, three to five-day-old agar cultures were harvested and resuspended in 20 ml of Hams F12 medium supplemented with 10% calf serum (Flow Laboratories, Irvine, UK) and kept in a microaerophilic environment for 3 days at 37^{o}C and kept at 4^{o}C. If no growth was observed after five days of incubation on GAB-CAMP agar at 37^{o}C harvested cells were defined as viable but non-culturable [Aleljung *et al.* 1996]. All bacteria were checked for the presence of spirals in the coccoid suspension and vice versa: the spiral form was not found in the coccoid suspension whereas a few coccoids were observed among the spiral suspension (less than 0.001%). Table 7 shows the origin and virulent status of *H. pylori* strains used in this thesis.

The cells were harvested in PBS, centrifuged at 3000 rpm for 10 minutes and resuspended in PBS to a final concentration of 10^9 colony-forming units (cfu)/ml.

Table 7. Origin and CagA / VacA expression of *H. pylori* strains.

Strains	Clinical manifestation	CagA	VacA
CCUG 17874[a]		+	+
25[b]		+	+
553/93	Gastritis	-	-
83/95	Duodenal ulcer	+	-
87/95	Duodenal ulcer	+	+
105/95	Atrophic gastritis	+	+
116/95	Gastritis	+	+
119/95	**Duodenal ulcer**	**+**	**+**
122/96	Duodenal ulcer	+	+
125/96	*Pyloric* ulcer	+	+
131/96	Gastritis	+	+

[a]: Strain CCUG 17874 was obtained from the Culture Collection of University of Gothenburg (CCUG); [b]: Strain 25 was isolated from Community Hospital of Helsingborg. All other strains are clinical isolates from University Hospital of Lund.

Animals and diets:

Six- to eight-week-old BALB/cA, C57BL/6, CBA/Ca and NMRI mice were used [Kaufmann *et al.* 1998]. Except in Paper III, mice were purchased from B&K Universal Company (Stockholm, Sweden), other BALB/cA and C57BL/6 mice were originally purchased from B&K company and bred in our animal house. The animals were housed with a 12-hour light-dark schedule, fed different diets (Table 8) and given water *ad libitum*. The rodent diets were kept in a special ventilated room for food storage in the animal house. All animal experiments in this thesis were carried out under the permission of Animal Care Committee at Lund University.

Table 8 Diets used in this thesis

Commercial diets	In paper	Fixed Formula			Heated
		Full fat soya	Soya meal	Fish meal	at ^{o}C
Standard No.1 (RM1, pelleted)[a]	I, II	–	+ (14%)	+	
Standard No.2 (RM2, expanded)[a]	II-VI	–	+ (24%)	–	140-150
Autoclavable diet (RMA)[a]	II	+	+ (20.8%)	+	105
Standard No.3 (SDS, RM3, pelleted)[b]	II	–	+ (11%)	+	

[a]: From B&K Universal Company, Sollentruna, Sweden; [b]: From SDS Company, London, UK.

Antioxidants:

Homogenized and dried cells of the unicellular green alga *Haematococcus pluvialis* (Fig. 8) rich in astaxanthin (2-3% wt/wt) and an algal meal without astaxanthin as control meal were obtained from AstaCarotene AB (Gustavsberg, Sweden). Vitamin C ($_{L}$-ascorbic acid) was purchased from ICN Biomedicals Inc. (Lund, Sweden). The algal meal and control meal were suspended in distilled water

and vitamin C was dissolved in distilled water just before use. The chemical structure of astaxanthin and vitamin C is shown in Fig. 9 and 10 respectively.

Animal model:

Mice were inoculated orally through a feeding tube (Outer Diameter=0.1cm) three times at 2-day intervals with 0.1 ml of either bacterial suspension (10^9 colony-forming units/ml) or PBS. The *H. pylori* infection was followed at different time points by sacrificing the animals (Fig. 11 A). The stomachs were collected for culture, PCR and histopathology. Blood were drawn for examining immune response. The scheme of inhibition *H. pylori* infection model is shown in Figure 11 B.

Culture:

Stomach biopsies were rinsed in PBS and smeared directly on GAB-Camp agar and incubated for 5-10 days in microaerophilic conditions at 37^{o}C. The presence of *H. pylori* on the culture plates was confirmed by urease, catalase, oxidase test, Gram staining and PCR analysis with HP urease primers [Nilsson *et al.* 1996].

Histopathology:

Stomach tissues were fixed in 10% buffered formalin (effective osmolar pressure 300 mosm/l) embedded in paraffin, 4 μm sections prepared, stained with haematoxylin and eosin, following standard procedures. The degree of inflammation was scored between 0-3 in the different parts of the stomach and duodenum (Table 9).

Table 9. The scale of inflammation score

Inflammation score	Describe in histopathology
0	Normal
1	few inflammatory cells
2	moderate inflammatory cells in several layers
3	high level of inflammation with nests containing more than 50 inflammatory cells, often more than three cell layers

ELISA and immunoblot:

Sera were examined for total antibodies (mainly IgG) to *H. pylori* by ELISA [Lelwala-Guruge *et al.* 1992]. Sera (diluted 1:200) 100 μl were added to each well

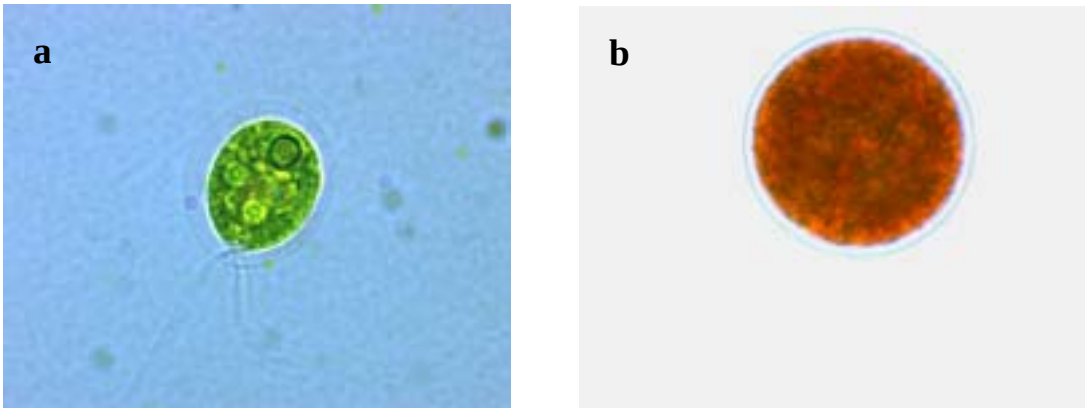


Fig. 8 (a) Vegetative actively growing *Haematococcus* cell (b) *Haematococcus* haematocyst that have accumulated astaxanthin as a result of nutrient starvation and sunlight. (Photos from Kjell Stalberg).

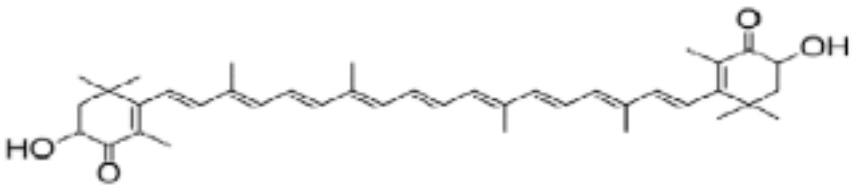


Fig. 9 Chemical structure of astaxanthin.

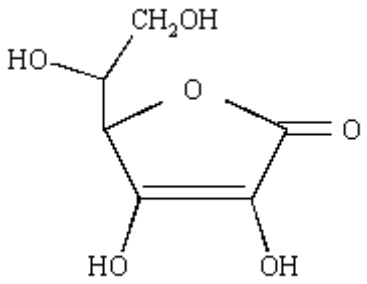


Fig. 10 Chemical structure of L-ascorbic acid

which had been coated with *H. pylori* antigen obtained from an acid glycine extraction. After incubation for 90 mins at 37°C, wells were washed and added with 100 µl of HRP-labeled

goat anti-mouse immunoglobulins (Dakopatts, Copenhagen, Denmark) diluted 1:2000 in the washing buffer. The plates were incubated for 60 mins at 37°C, substrate and stopping solution were added and absorbance values at 450 nm were registered in a spectrophotometer.

Sera diluted 1:50 in washing buffer (10 mM Tris base, 30 mM NaCl, 1 mM NaN_3, pH 10.2 and 0.1% Tween 20) were incubated with strips which were received

from SDS-PAGE and blot using glycine extraction of *H. pylori* overnight on a shaker at 4°C [Nilsson *et al.* 1997]. Anti-mouse Immunoglobulins labeled with HRP (dilute 1:600) were added for 3 hours on a shaker at 4°C and developed 30-40 minutes with 50 mM NaAc containing 0.02% carbazole and 0.3% H_2O_2 in room temperature.

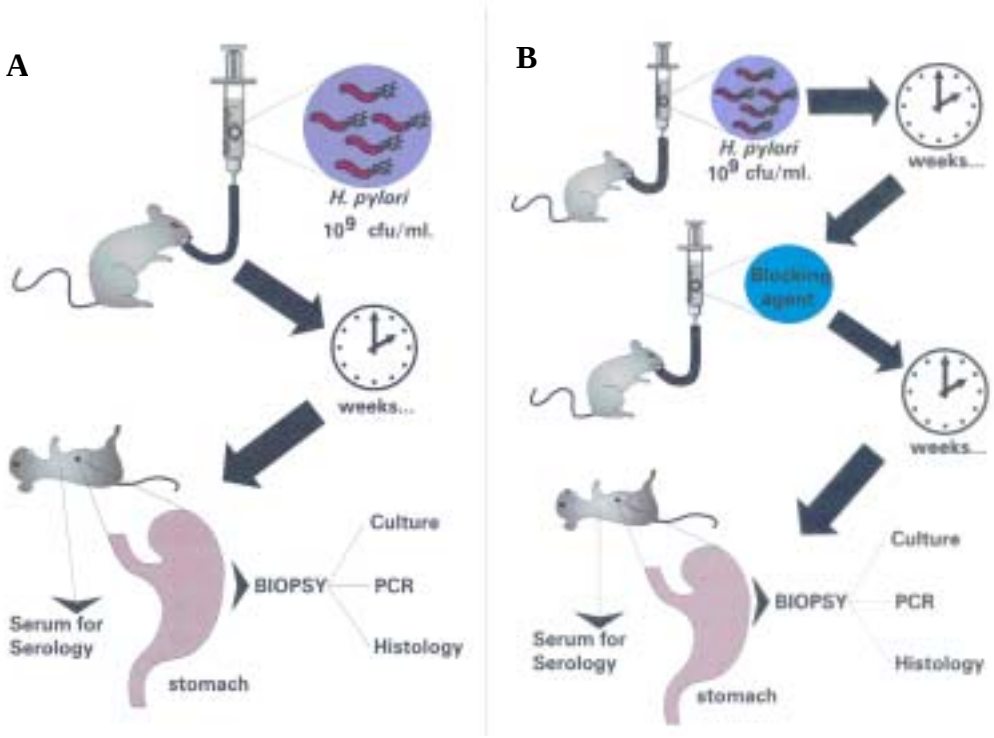


Fig. 11 A) *H. pylori* infection mouse model; and B) Inhibition of *H. pylori* infection in a mouse model.

DNA extraction and PCR conditions:

DNA was extracted from frozen homogenates of stomach biopsies from control and *H. pylori*-infected mice. 100 μl of homogenate were centrifuged 12,000 g for 5 minutes and resuspended in TNE buffer 380 μl with 1% Triton X-100 and 0.5 mg/ml lysozyme. The samples were incubated at 37°C for 30 mins, added proteinase K to 1 mg/ml and incubate overnight at 37°C. DNA was isolated by extracting twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform. DNA was precipitated with 0.3 M sodium acetate and 2 volumes of absolute ethanol and centrifuged as described above, rinsed in 70% alcohol, dried in a

speed vacuum [Kong *et al.* 1996]. The DNA pellet was dissolved in 50 µl water plus tween and stored at -20^{o}C until the PCR is done.

HPU primers (Scandinavian Gene Synthesis, Köping, Sweden), based on a urease A gene sequence of *H. pylori*, was used [Nilsson *et al.* 1996].

RAPD-PCR analysis:

Individual colonies from GAB-Camp agar were isolated by sterile toothpicks and suspended in 50µl of 10mM EDTA, pH 8.0 in Eppendorf tubes. Freshly prepared cracking 2X buffer (50 µl, 0.67 M NaOH, 10% SDS, 20% sucrose) were added and resuspended by vortex. All tubes were incubated at 70^{o}C for 5 minutes and 1.5µl of 4M KCl added after the tubes were cooled to room temperature. Samples were then put on ice for 5 minutes and centrifuged at 12,000g for 3 minutes at 4^{o}C. DNA was precipitated by adding 1/10 volume of 4M LiCl to the supernatant and 2.5 volumes of absolute ethanol incubated in -20^{o}C for 20 minutes. DNA was pelleted by centrifugation at 12,000g for 10 minutes, rinsed by 70% ethanol and resolved in 50µl PCR grade water.

A mixture of two primers 1254 and 1283 were used [Vandamme *et al.* 1995]. A conventional PCR-buffer was used for amplification; consisting of 10mM Tris-HCl (pH 8.3), 50mM KCl, 3mM $MgCl_2$, 0.01% BSA, 0.2mM of each deoxynucleotide-triphosphosphate, 0.8µM of each primer and Taq polymerase (1.875 U; Boehringer Mannheim, Mannheim, Germany). PCR condition consisted of 5 cycles of 5 min 94^{o}C, 5 min 36^{o}C and 5 min 72^{o}C followed by 30 cycles of 1 min 94^{o}C, 1 min 36^{o}C and 2 min 72^{o}C with a final elongation step of 10 min 72^{o}C. Amplified products (15µl) were analyzed in agarose electrophoresis (1.5% gels) and visualized by ethidium bromide staining. DNA amplification patterns were considered identical when no variation was observed in the DNA banding patterns.

Carotenoid and astaxanthin analysis:

Stomach samples were homogenized and extracted in acetone. The acetone extracts were pooled, mixed vigorously with cyclohexane (1:1) and approximately 200-400 µl distilled water to obtain phase separation. The samples were then centrifuged and the concentration of carotenoids recovered in the hexane phase determined by measuring the absorbency at 474 nm with a Spectronic 601

spectrophotometer (Milton Roy Co., USA). An extinction coefficient of μg/kg was used for calculations.

The carotenoid composition was determined by high pressure liquid chromatography (HPLC) after evaporation of the cyclohexane extract to dryness with nitrogen and dissolving the carotenoids in chloroform:methanol (2:1). The HPLC system (Merck-Hitachi) consisted of a L6200A Intelligent pump, a D-2000 injector and a L4200 Vis/UV-detector set at 474 nm with 0.1 absorbency units at full scale. External internal carotenoid standards (astaxanthin and canthaxanthin, 99% pure, Hoffman-La Roche Ltd, Denmark) were employed to check the recovery of carotenoid during extraction and the reproducibility of the analytical methods applied. All the solvents and chemicals used were of analytical grade and purchased from Merck, Germany.

Astaxanthin analysis of AstaCarotene Algal meal:

3 mg of algal meal was dissolved in 200 μl water for 15 mins and was added 10 ml acetone. The sample was homognized if larger particles or clumps were present and left for 2 hours in a dark and cold place. The sample was centrifuged and the absorbance of the supernatant was measured at 474 nm. The concentration of carotenoids in the sample is linear between absorbance 0.05 to 0.9. Calculation of carotenoids (mg) in the sample A_{474}/193 X10 and 90% of the carotenoids in the algal meal is astaxanthin.

Lipid peroxidation assay:

Mice stomach tissue was homogenized in 20mM Tris-HCl, pH 7.4 to 10% (w/v). 200 μl of homogenate supernatant were tested for malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE) by a lipid peroxidation assay kit from CALBIOCHEM® (San Diego, California, USA). The colorimeters were measured at absorbency at 586 nm and tissue lipid peroxidation was calculated as μmol/g tissue.

In vitro inhibition test:

The minimum inhibitory concentrations (MICs) were determined as elsewhere [Ohta *et al.* 1999] that each preculture containing 10^3 cells was plated onto GAB-Camp agar with or without various concentrations of algal meal (0.375 to 20 mg/ml), algal meal without astaxanthin (5 mg/ml) or vitamin C (0.5-4 mg/ml). The surviving

cells were counted on the plate as colonies and the MIC was defined the concentration leaving no survivors after 5-10 days incubation under microaerophilic condition.

Statistical Analysis:

The Mann-Whitney *U* test was used for analysis of colonization, inflammation distribution and changes of immune response. The level of significance was chosen as $p < 0.05$.

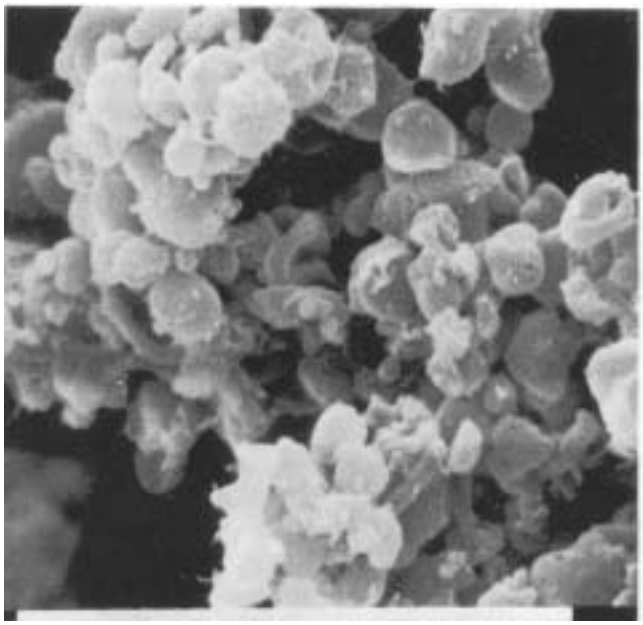
Results and Discussion

Paper I:

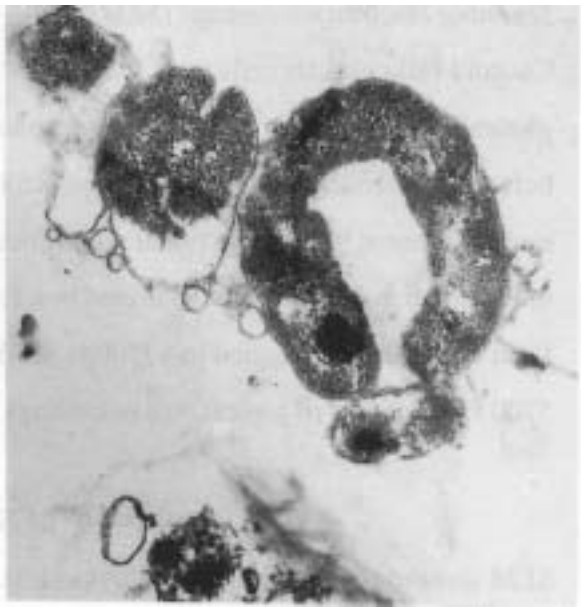
A *H. pylori* infection was followed up to 30 weeks in BALB/cA mice inoculated by both spiral and coccoid forms. Both forms of *H. pylori* strain CCUG 17874, 25 and 553/93 induced a significant high inflammation score in the murine stomach (Paper I, Fig. 2 and 3); strain 553/93, a fresh isolate, caused a significantly higher inflammation score than the other two strains (Paper I, Fig. 4). An immune response was seen after 4 weeks in mice infected with the spiral form of strain CCUG 17874 and after 16 weeks in mice infected with coccoids or the other two strains (Paper I, Fig. 6 and 7). Histopathological studies showed type B gastritis with infiltration of polymorphonuclear leukocytes (PMN) and lymphocytes and persisted during the entire experiment of 30 weeks. In contrast, an uninfected control group and formalin-killed *H. pylori* inoculated control group showed no evidence of gastritis.

H. pylori infection is worldwide and once acquired, it becomes chronic and probably persists for life if untreated [Blaser *et al.* 2001]. Multiple lines of evidence demonstrate a causal role of *H. pylori* in the chronic inflammatory process [Ernst 1999]. Early studies showed that oral challenges with *H. pylori* resulted in transient to chronic gastritis in nude and euthymic mice, gnotobiotic piglets and monkeys [Karita *et al.* 1991; Engstrand *et al.* 1990; Dubois *et al.* 1994]. *H. pylori* exist in two forms, spirals and coccoids. The coccoid forms have been described as ‘VBNC’ cells and suspected for the survival of the bacterium outside the human host [Benaissa *et al.* 1996; Andersen *et al.* 2000]. Cellini reported that these coccoids could revert to spiral forms in mice and induce a humoral immune response [Cellini *et al.* 1994]. These forms are found more frequently and in larger numbers in cases of adenocarcinoma than in benign ulcers [Chan *et al.* 1994]. In a parallel study of morphologic conversion from spirals to coccoids of *H. pylori* strain 553/93, we found preserved subcellular structures and intact double membranes in combination with degenerative forms suggesting that some of the coccoids are viable (Fig. 12) [Willen *et al.* 2000]. Both spiral and coccoid forms gave a significant increase in inflammatory cells in murine stomach and produced a systemic antibody response.

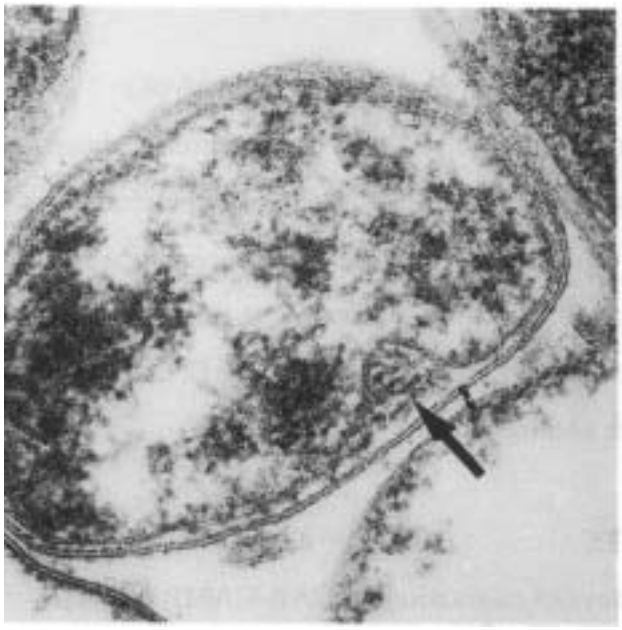
Fig. 12 Morphological conversion of *H. pylori* strain 553/93 from spirals to coccoids.



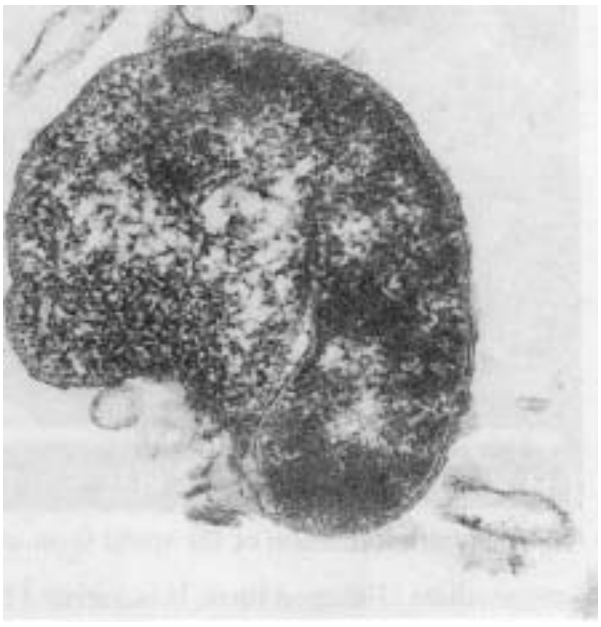
A: Clusters of *H. pylori* strain 553/93 after 5 days cultivation on GAB-Camp agar with coccoids and some debris. No spirals are detected. SEM x 5,700 (original magnification).



B: U-formed spiral bacteria. Note cytoplasmic vesicles, polar density and light areas, supposedly polyphosphate-rich areas. Diameter 1.34 μm. TEM x 21,000 (original magnification).

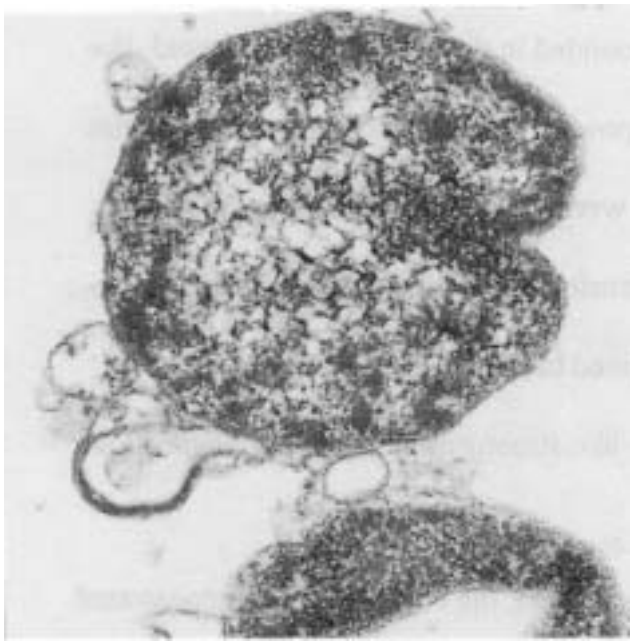


C: Spiral form with separation of inner and outer membrane by the periplasmic space with accumulation of dense granular material (Arrow). TEM x 105,000 (original magnification).

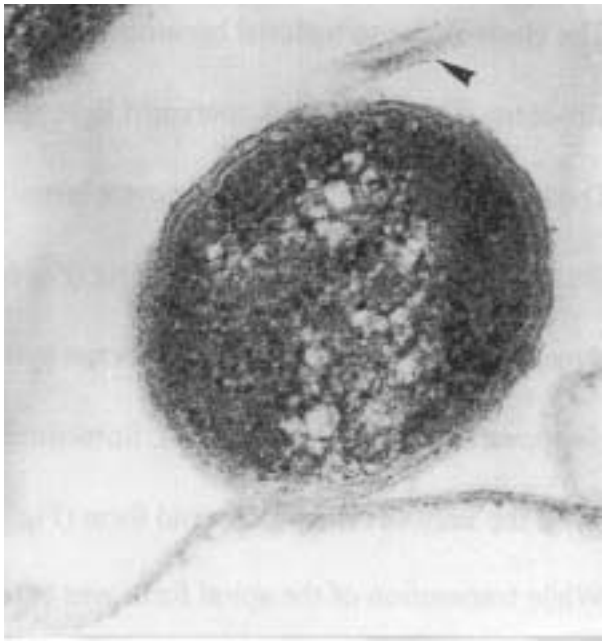


D: Partly formed coccoid. Condensation of sense material in one part of the bacteria, double membranes and shedding of cytoplasmic vesicles. TEM x 52,000 (original magnification).

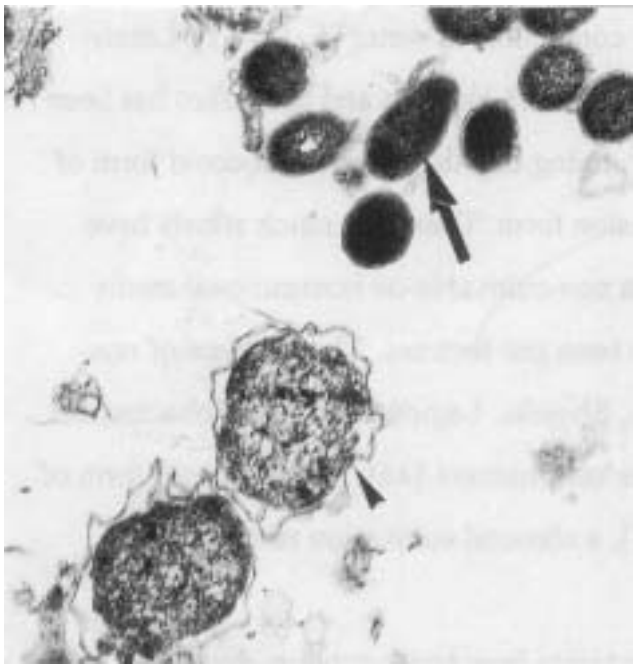
Fig. 12 Morphological conversion of *H. pylori* strain 553/93 from spirals to coccoids (Continued).



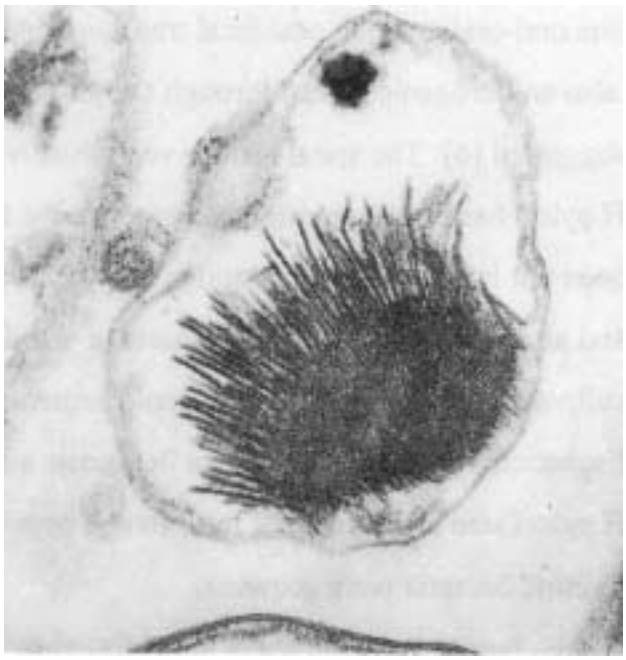
E: Nearly finished *H. pylori* coccoid. Note light areas and cytoplasmic vesicles sometimes containing dense material. TEM x 52,000 (original magnification).



F: Fully developed *H. pylori* coccoid. Note double membranes, dense and light areas and part of flagella (Arrow). TEM x 105,000 (original magnification).



G: Mixture of transected spiral *H. pylori* (Arrow) (Diameter 0.41-0.45 μm) and coccoid forms (Arrowhead) (1.04-1.16 μm). TEM x 15,500 (original magnification).



H: Coccoid with morphologically clear degeneration and fragmentation of membranes and central dense area. TEM x 73,000 (original magnification).

Paper II:

Dietary factors have been considered important for developing gastric cancer in human epidemiological studies and in animal models [Correa 1992a; Kanke *et al.* 1996]. The prevalence of *H. pylori* is associated with dietary factors such as high intake of salty food and soybean products and low intake of vitamin C or carotenes in various populations [Tsugane *et al.* 1994; Goodman *et al.* 1997; Shinchi *et al.* 1997; You *et al.* 2000].

Our data indicate that various mouse diets influence culture results of *H. pylori* from infected BALB/cA mice. In experiment 1, the infection was carried out in animals already on a specific diet. *H. pylori* was isolated 100% from inoculated mice based on the RM2 diet compared to only 10-20% based on other diets (Paper II, Table 2). In experiment 2, infected mice were changed to different diets and the recovery rate of *H. pylori* decreased to 50-60% on the RM1, RMA and SDS diets compared to 90% in RM2 (Paper II, Table 3). Mice fed with the RM2 diet also showed the highest inflammation scores for the four commercial diets indicating that the higher density of *H. pylori* caused the more severe inflammation. The four diets examined show few differences in the contents or amounts of the different compounds (Table 8). The lack of fish meal and the production process for expanded food (heated at 140-150^{o}C) might partly explain why RM2 sustains *H. pylori* infection the best. Fish oils and polyunsaturated fatty acids have shown a bactericidal activity to *H. pylori in vitro* and fish oil enriched vitamin E-deficient diets suppress lethal *Plasmodium yoelii* infection in athymic mice [Thompson *et al.* 1994; Drago *et al.* 1999; Taylor *et al.* 1997]. While we were unable to pinpoint the exact dietary factor for *H. pylori* infection in this mouse model, it is valuable to optimize the diet to standardize mouse models of *H. pylori* infection. The diet without fish meal and expanded at 140-150^{o}C is selected for our further experiments.

Paper III:

To further characterize and optimize a mouse model for *H. pylori* type B gastritis we investigated the infection in 3 inbred and 1 outbred mouse strains with a mixture of 9 fresh isolated strains (Paper III, Table 1). A total of 577 single colonies of *H. pylori* were collected from culture positive animals and identified as one of the

original inoculated strains. One dominant strain colonized 66 of 75 animals (88%), whereas 8 (10.7%) mice had 2 strains and only 1 animal (1.3%) was found with 3 strains in its stomach. 522 of 577 isolates (90.5%) match the original strain 119/95 by RAPD-PCR analysis (Paper III, Table 3). Inflammation scores were much higher for *H. pylori* infected animals than non-infected controls for all four mouse strains ($p<0.01$) and the inflammatory cell infiltration were mainly granulocytes during the entire infection (Paper III, Fig. 2 and 3). The four mouse strains demonstrated severe gastritis 16 weeks post-inoculation of *H. pylori* with minor differences between strains. A specific immune response to *H. pylori* was detected already after 4 weeks of infection by immunoblot (Paper III, Fig. 4) and the four mouse strains were ranked from highest immune response as follows: NMRI > BALB/cA > C57BL/6 > CBA/Ca mice.

Our findings that most mice were infected with 1 strain and only a few animals were infected with 2 or 3 strains which were recovered only during the early phase of infection indicate a time dependent decrease of strains which might reflect that strains possess different colonization abilities. Dubois *et al* [Dubois *et al.* 1996] investigated isolates from 3 rhesus monkeys infected with 2 *H. pylori* strains and only one of the strains could be re-isolated. The Sydney mouse passaged strain SS1 was the only strain isolated from mice inoculated by a mixture of 4 fresh clinical isolates [Lee *et al.* 1997]. In a recent report, 2 *cag*A-/*vac*A+ and 1 *cag*A+/*vac*A+ *H. pylori* strains were found to “take over” in a mixed infection in outbred mice [Sturegard *et al.* 2001].

Host dependent gastric atrophic changes to a *H. felis* or *H. pylori* infection was found in mice [Sakagami *et al.* 1996; Lee *et al.* 1997]. In SJL, C3H/He, DBA/2, and C57BL/6 *H. felis* infected mice, a severe to moderate chronic active gastritis in the body of stomach developed while BALB/c and CBA mice, only got mild gastritis in the antrum. *H. pylori* infected C57BL/6 mice exhibited moderate to severe body atrophy, whereas C3H/He mice showed little development of atrophy [Sakagami *et al.* 1996]. The colonization levels of *H. pylori* strain SS1 varied among C57BL/6, Balb/c, DBA/2 and C3H/He mice and both C57BL/6 and BALB/c mice developed chronic active gastritis to severe atrophy during 8 months [Lee *et al.* 1997]. In our study C57BL/6 and BALB/cA mice showed higher inflammation scores at 4 and/or 10 weeks post-inoculation (p.i.) compared to CBA/Ca and NMRI mice.

Strain 119/95 is selected for further development of our mouse model. The C57BL/6 and BALB/cA mice were used for further *H. pylori* infection studies.

Paper IV:

C57BL/6 mice (n=5) inoculated with the *H. pylori* mouse passaged strain 119/95p and the infection was followed up to 13 months. Two main changes were found in histopathology: squamous papillomalike structures with growth capacity to some extent mimicking a highly differentiated squamous cell carcinoma and blast-like high-grade lymphoma positive for B-cell markers showing highly abnormal cells and mitosis (Paper IV, Fig. 1). The tumor cell population destroyed the local mucosa and muscular tissue and was seen growing outside the gastric tissue. Moreover a heavy infiltration of the same cell populations were seen in the liver, spleen and kidney tissue, clearly demonstrated the malignant features of the lymphoma. The normal control animals (n=2) showed no pathological changes in any of these organs.

Our previous findings showed that C57BL/6 mice infected with *H. pylori* 119/95p for 6 months developed severe gastritis and atrophic changes [Wang X *et al.* 1998b]. *H. pylori* usually creates a low-grade (so called MALT) lymphoma in humans as well as in animal models [Hussell *et al.* 1993; Isaacson 1994; Enno *et al.* 1995]. There are also reports on high-grade and a mixture of high and low grade lymphoma in humans and mice [Isaacson 1996; Enno *et al.* 1998; Isaacson 1994]. De novo high-grade lymphoma occurs sometimes [Isaacson 1996]. *H. pylori* can be identified in more than 90% of cases of gastric MALT lymphomas [Chan *et al.* 1990; Wotherspoon *et al.* 1993] and fewer in more advanced cases [Peng *et al.* 1998]. This suggests that *H. pylori* is more closely associated with the precursor or initial genesis of MALT lymphoma [Nakamura *et al.* 1998]. High-grade MALT lymphoma transformation may be more likely to occur following infection by CagA positive strains of *H. pylori* [Peng *et al.* 1998]. The strain used in our study was both CagA and VacA positive. Grading according to Wotherspoon *et al* [Wotherspoon *et al.* 1993] revealed definite or suspected local gastric lymphoma with a B-cell pattern. However in humans, some cases of T-cell gastric lymphomas have been reported [Foss *et al.* 1999; Isaacson 1994]. *H. pylori* can be found in combination with gastric carcinoma and lymphoma [Eidt *et al.* 1995]. In this study we found high-grade B cell lymphoma derived from MALT-lesions together with highly proliferative squamous

cells with a papillomatous feature and expanding borders, however with no definite carcinoma.

In our recent studies lymphoepithelial lesions (LEL) were found in C57BL/6 and BALB/cA mice infected with *H. pylori* strain 119/95 and G50 but not with strain SS1 after 23-month infection. There was 1 case of hepatoma found in strain 119 infected C57BL/6 mouse after a 23 months infection. These data confirm that our mouse model infected with strain 119/95, a CagA positive and vac toxin producing strain, is a suitable model to study of gastric lymphoma development and to compare future treatment strategies.

Paper V:

With the progression of antibiotic resistance of *H. pylori*, novel non-antibiotic strategies to treat are needed [Dajani *et al.* 2000]. Rebamipide, a gastroprotective drug, was found to scavenge ROS and attenuate neutrophil activity and their production of inflammatory cytokines stimulated by *H. pylori* [Danielsson *et al.* 1998; Iinuma *et al.* 1998; Arakawa *et al.* 1998]. Our previous study showed that dietary factors influence *H. pylori* infection in our mouse model [Paper II]. These points lead us to study the effect of antioxidants on *H. pylori* infection in the mouse model.

An astaxanthin-rich algal meal and vitamin C inhibit *H. pylori* growth *in vitro* while algal meal without astaxanthin did not show this effect (Table 10). Mice treated with the astaxanthin-rich algal meal or vitamin C showed significantly lower colonization and inflammation scores than control mice infected with *H. pylori* or treated with a meal lacking astaxanthin (Paper V, Fig. 1 and 2). All astaxanthin-rich algal meal- or vitamin C-treated mice showed significant decreases in lipid peroxidation compared with levels in untreated and control-meal-treated animals (Paper V, Fig. 4). Among the three doses of astaxanthin tested, the highest dose (100mg/kg) showed the best effect to reduce bacterial load and gastric inflammation.

H. pylori infection has been associated with a decreased level of vitamin C and other major antioxidants (eg. β-carotene) in human gastric tissues [Correa *et al.* 1998; Phull *et al.* 1995]. The infected individuals show a high oxidative stress and high level of ROS in the gastric mucosa and an increased gastric antioxidative capacity after

Table 10 MICs values against *H. pylori*[a].

Compounds	MICs
Algal meal (astaxanthin content)	0.3125-2.5 mg/ml (6.25-50 μg/ml)
Algal meal without astaxanthin	>5 mg/ml
Vitamin C	0.5-2 mg/ml

[a] 10 strains of *H. pylori* were tested.

eradication of *H. pylori* [Khaled *et al.* 1998]. A recent study on formation of pro- and anti-oxidants to *H. pylori* infection in Mongolian gerbils showed an increase in the level of lipid peroxidation and an activated glutathione turnover [Suzuki *et al.* 1999]. Vitamin C has been reported to inhibit *H. pylori* both *in vitro* and *in vivo*, but not vitamin E [Zhang HM *et al.* 1997]. The inhibitory effect appears to be specific for *H. pylori* and the closely related *Campylobacter jejuni*, as various *E. coli* strains, *Salmonella,* and *Vibrio* species were unaffected. Patients with *H. pylori* chronic gastritis received a high dose of vitamin C (5g per day) daily for 4 weeks resulted in a *H. pylori* eradication in 30% [Jarosz *et al.* 1998]. The mechanism of the inhibitory action of vitamin C on *H. pylori* is not known. A recent study on *Streptococcus pneumoniae* suggested that vitamin C may compress or retard bacterial invasion by directly inhibiting the bacterial spreading engine hyaluronate lyase [Li *et al.* 2001].

Astaxanthin has superior antioxidative activity to most of the hydrophobic antioxidants and is most effective to stimulate immune defenses [Jyonouchi *et al.* 1996; Naguib 2000; Miki 1991]. Our study is the first demonstration of an antimicrobial activity of an algal meal against *H. pylori* and associated gastric inflammation [Wadström *et al.* 1997]. The possible mechanisms of astaxanthin-rich algal meal action *in vivo* could be: (i) a antimicrobial effect on *H. pylori*, (ii) that it neutralizes ROS in the mucosa and attenuates the inflammation, (iii) its algal cell walls may adhere to gastric epithelium competitively, and (iv) it may inhibit the infection through an altered immune response [Paper VI].

Paper VI:

In this study we demonstrated, that *H. pylori* infected BALB/cA mice treated with the antioxidant astaxanthin-rich algal meal decreased the bacterial load and

inflammation scores in the stomach, and shift in the cytokine release profile of cultured splenocytes *in vitro*.

BALB/cA mice infected with *H. pylori* display a Th1 T-cell response with a high release of IFN-γ and no IL-4 as described in man [Czinn *et al.* 1997; Walker *et al.* 1998; Lindholm *et al.* 1998; Sommer *et al.* 1998]**.** However, splenocytes from astaxanthin-rich algal meal treated animals showed a significant increase in IL-4 release, indicating a shift towards a Th2 T-cell response**.** An excessive Th1 response driven by the infection with *H. pylori* may favor the development of a cell-mediated immune response and an inflammatory (cytotoxic) damage of the epithelium [Czinn *et al.* 1997]. Our data support that an immunomodulation can enhance a protective and nondestructive response against *H. pylori*, attenuating the infection and the subsequent gastric inflammation as described previously [Mohammadi *et al.* 1997]. The observed shift of the Th1/Th2-balance following treatment is probably due to a down-regulation of Th1-cells and an up-regulation of Th2-cells by astaxanthin [Jyonouchi *et al.* 1996]. The antioxidant rebamipide was reported to regulate cytokine responses by human peripheral blood mononuclear cells with *H. pylori* infection [Aihara *et al.* 1998] and it might contribute to disease regression and bacterial eradication. Astaxanthin-rich algal meal could be effective the same manner.

In this model the switch from a Th1 type of response to a mixed Th1/Th2 type of response is experimentally induced during an ongoing infection. To our knowledge, it is the first time a shift in a T-lymphocyte response has been achieved during an ongoing infection. Another possible mechanism of action is that astaxanthin acts as antioxidant scavenging ROS involved in the pathogenesis of the *H. pylori* induced chronic gastritis as discussed in the paper V.

Conclusions and future prospects

Both spiral and coccoid forms of *H. pylori* were found to induce gastritis and produce a systemic immune response in BALB/cA mice following 30 weeks of infection. Dietary factors play an important role in *H. pylori* infection and we have established an optimized diet to facilitate the infection in a mouse model. The strain 119/95 was the most common strain colonizing all 4 mouse strains and it does not seem to have any host restriction. *H. pylori* 119/95 and C57BL/6 and BALB/cA mice were characterized for further development of our mouse model.

This mouse model with infection by CagA-positive, vacuolating-toxin-producing *H. pylori* 119/95 is suitable for use in the study of lymphoma development and squamous cell papilloma with proliferative features. Antioxidants such as an astaxanthin-rich algal meal and vitamin C inhibit *H. pylori* both *in vitro* and in infected mice. Inhibition of this infection by the astaxanthin-rich algal meal was associated with a switch in the systemic T-helper cell response. These results suggest that antioxidants may become a new strategy for treating *H. pylori* gastritis in humans [Wadström *et al.* 1997]. We have the optimized mouse model to test new treatment strategies for *H. pylori* infection.

Animal models are very important in the investigation of disease processes, establishing new experimental therapy and vaccine development against *H. pylori* infection. With the complete genome of mouse strain C57BL/6 and other laboratory mouse strains (129X1/SvJ, DBA/2J and A/J) available in the near future [Marshall 2001], mouse models will be most efficiently used to study the pathogenesis of *Helicobacter* infection. *H. pylori* has two complete genome sequences available [Tomb *et al.* 1997; Alm *et al.* 1999] and genomic research will allow us to ascertain how the organism functions through biochemical pathways and the role played by the *cag*A pathogenicity island. A protein-protein interaction map of *H. pylori* was constructed and over 1,200 interactions were identified between proteins, connecting 46.6% of the proteome [Weeks *et al.* 2000; Scott *et al.* 2000]. To date the goal of deriving *Helicobacter*-specific therapies from genome analysis has not been achieved in clinical practice, but several genes and metabolic pathways of the pathogen are recognized as potential targets for drug development.

With the progression of antibiotic resistance and the challenge of re-treatment following treatment failures of *H. pylori* infection, novel non-antibiotic approaches including new drugs, vaccines, probiotics and anti-adhesion compounds are needed. Antioxidants such as vitamin C and astaxanthin demonstrate inhibitory effects against *H. pylori in vitro* and in animal models [Zhang HM *et al.* 1997; Paper V]. Dietary supplements with β-carotene and vitamin C may be coupled to additional antimicrobial therapy may prevent *H. pylori*-associated gastric cancer [Mannick *et al.* 1996]. An astaxanthin-rich algal meal has been tested as a treatment for *H. pylori* positive NUD patients, where 1 out of 10 patients was completely cured from *H. pylori* infection [Borody *et al*, submitted for publication]. A second clinical trial is now underway in Kaunas, Lithuania. Treatment with this astaxanthin-rich algal meal and vitamin C in a *H. pylori*-induced lymphoma mouse model would provide us with more evidences as to whether antioxidants could provide a new therapeutic area for *Helicobacter* infection. There are 4,000 purified diets available for laboratory animals in Research Diets Inc., which may help researchers to find out which specific factor(s) could be vital for *H. pylori* infection.

In addition, the ability to adhere to gastric cells may be essential for sustained infection and *H. pylori*-induced disease [Falk *et al.* 2000; Wadström *et al.* 1996]. Blocking of adhesins could represent a potential target for therapy. One anti-adhesion compound 3'-sialyllactose sodium salt (3'SL), an oligosaccharide that occurs naturally in human and bovine milk, and milk glycoconjugates, have shown an inhibitory effect on *H. pylori* infection in rhesus monkeys and mice [Mysore *et al.* 1999; Wang *et al.* 2001]. Some gastric mucoadhesive drug delivery systems are very promising for eradication of *H. pylori*, such as gelatin microsphere has been suggested for a candidate delivery system [Wang J *et al.* 2000]. New approaches are needed for future developments in *H. pylori* therapy.

H. pylori infection can only induce gastric adenocarcinoma in mice when chemical carcinogens are used simultaneously [Lee 1999]. *H. felis* infection in gastrin transgenic mice is associated with progression of gastric cancer [Wang TC *et al.* 2000]. It would be more interesting to study the *H. pylori* infection in these gastrin transgenic mice strains. Such transgenic mouse models may become essential for studying relationship between *H. pylori* and gastric cancer [Falk *et al.* 2001]. Moreover, since gastric cancer is associated with *p*53 over-expression and *H. pylori*

infection [Murakami *et al.* 1999], we are continuing to optimize *H. pylori* infection in a *p*53 transgenic mouse model. Experiments are going on in our laboratory to further optimize this mouse model in conjunction with bile acid.

Sammanfattning på svenska

Helicobacter pylori är en spiralformad Gramnegativ human-specifik patogen, som kan penetrera magsäckens slemlager och kolonisera epitelskiktet. *H. pylori* infektion är en högriskfaktor för utvecklandet av kronisk gastrit, magsår och ventrikelcancer.

Både aktivt växande spiralformer av *H. pylori* och dess runda viloformer framkallar kronisk magkatarr (gastrit) i BALB/cA möss och orsakar ett immunologiskt svar med antikroppsbildning. Preliminära försök visade att infektionen påverkades starkt av den diet som djuren utfordrades med. En diet utan fiskmjöl gav den starkaste inflammationen och gastritbilden vid mikroskopisk undersökning. Infektion med en blandning av nio olika *H. pylori* stammar visade att en stam, 119/95, oftast snabbt konkurrerade ut övriga stammar. Denna stam har därför utvalts för vidare studier. I en annan musstam framkallade den kroniska infektionen ofta en specifik tumör, s.k. MALT- lymfom, medan djuren ej utvecklade magcancer, som är en vanligare *H. pylori*-associerad tumörform hos människa. *H. pylori*-infektion framkallar bildning av fria syreradikaler, som kan neutraliseras av antioxidantia såsom vitamin C. I ett senare försök visades att såväl vitamin C som astaxantin (en annan potent antioxidantia) kunde hämma infektionen och inflammationen.

Dessa lovande resultat utnyttjas nu till att ta fram en ny behandlingsprincip för *H. pylori*- framkallad magkatarr hos människa. Slutligen har immunologiska studier i möss och på muslymfocyter visat att astaxantin framkallar en förändrad balans i de två huvudpopulationerna av dessa celler, vilket troligen underlättar för immunapparaten att utrota infektionen. Denna upptäckt kan förklara varför en obehandlad *H. pylori*-infektion utvecklas till en kronisk infektion med följdsjukdomar som magsår, MALT-lymfom och magcancer.

Acknowledgment

I would like to express my sincere gratitude to:

Professor Torkel Wadström, my supervisor, for introducing me to the field of *Helicobacter pylori* research, for his scientific advice and support, for sharing his great knowledge with me.

Professor Roger Willén, my co-supervisor, for great support, being so positive to me, and endless encouragement.

Associate professor Åsa Ljungh, for her kindness, support and providing her apartment to us in Lund.

Dr. Åke Lignell, for his kindness, understanding and great support.

Professor Stig Bengmark, my former supervisor, for inviting me to study in Lund University.

Dr. Pär Aleljung, for introducing me in the lab and giving me so much support at the beginning of my study.

Dr. Ingrid Nilsson, for taking care of all problems in the lab and support whenever I need.

All the co-authors of the articles included in this thesis.

Elisabeth Elfving for analyzing carotenoids of mice samples and preparing the control algal meal for the experiments.

All my colleagues, Hans-Olof, Mads, Håkan, Erik, Séan, Waleed, Elisa, Meeme, Ima, Dornota, Naoko, Tobias, Rolf, and all former colleagues in TW lab, for collaboration and friendship.

President Xishan Hao, my former directors and colleagues in Tianjin Cancer Hospital and Institute, for their encouragement and friendship.

My mother for her endless love, enormous support and encouragement, and helping me taking care of my baby daughter during this last year. My father for great support, love and endless encouragement. My mother-in-law for love.

Finally, but not least, Daiqing Li, my husband, for endless love and always thinking me first. Yinuo, our lovely daughter for bringing a lot of happiness to the family.

This study was supported by grants from the Swedish Medical Research Council (16X04723), the Swedish Forestry Agriculture Research Council (50.0497/98), Kungliga fysiografiska sällskapet in Lund and John and Augusta Perssons Foundation

in Lund, the Medical Faculty, Lund University and AstaCarotene AB, Gustavsberg, Sweden, CN (Curt Nicolin) foundation.

References

Adachi K, Ishihara S, Hashimoto T, Hirakawa K, Niigaki M, Takashima T, Kaji T, Kawamura A, Sato H, Okuyama T, Watanabe M and Kinoshita Y (2000). "Efficacy of sucralfate for *Helicobacter pylori* eradication triple therapy in comparison with a lansoprazole-based regimen." Aliment Pharmacol Ther 14(7): 919-22.

Aihara M, Imagawa K, Funakoshi Y, Ohmoto Y and Kikuchi M (1998). "Effects of rebamipide on production of several cytokines by human peripheral blood mononuclear cells." Dig Dis Sci 43(9 Suppl): 160S-166S.

Aldoori WH, Giovannucci EL, Stampfer MJ, Rimm EB, Wing AL and Willett WC (1997). "Prospective study of diet and the risk of duodenal ulcer in men." Am J Epidemiol 145(1): 42-50.

Aleljung P, Nilsson HO, Wang X, Nyberg P, Morner T, Warsame I and Wadström T (1996). "Gastrointestinal colonisation of BALB/cA mice by *Helicobacter pylori* monitored by heparin magnetic separation." FEMS Immunol Med Microbiol 13(4): 303-9.

Alm RA, Ling LS, Moir DT, King BL, Brown ED, Doig PC, Smith DR, Noonan B, Guild BC, deJonge BL, Carmel G, Tummino PJ, Caruso A, Uria-Nickelsen M, Mills DM, Ives C, Gibson R, Merberg D, Mills SD, Jiang Q, Taylor DE, Vovis GF and Trust TJ (1999). "Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*." Nature 397(6715): 176-80.

Andersen LP, Dorland A, Karacan H, Colding H, Nilsson HO, Wadstrom T and Blom J (2000). "Possible clinical importance of the transformation of *Helicobacter pylori* into coccoid forms." Scand J Gastroenterol 35(9): 897-903.

Andersen LP, Norgaard A, Holck S, Blom J and Elsborg L (1996). "Isolation of a "*Helicobacter heilmanii*"-like organism from the human stomach." Eur J Clin Microbiol Infect Dis 15(1): 95-6.

Andersen LP and Wadström T (2001). Basic bacteriology and culture. *Helicobacter pylori*: Physiology and Genetics. Mobley HLT, Mendz GL and Hazell SL. Washington, D.C., ASM press: 27-38.

Arakawa T, Kobayashi K, Yoshikawa T and Tarnawski A (1998). "Rebamipide: overview of its mechanisms of action and efficacy in mucosal protection and ulcer healing." Dig Dis Sci 43(9 Suppl): 5S-13S.

Babbs CF and Steiner MG (1990). "Simulation of free radical reactions in biology and medicine: a new two- compartment kinetic model of intracellular lipid peroxidation." Free Radic Biol Med 8(5): 471-85.

Bae EA, Han MJ and Kim DH (1999). "In vitro anti-*Helicobacter pylori* activity of some flavonoids and their metabolites." Planta Med 65(5): 442-3.

Bagchi D, Bhattacharya G and Stohs SJ (1996). "Production of reactive oxygen species by gastric cells in association with *Helicobacter pylori*." Free Radic Res 24(6): 439-50.

Bamford KB, Fan X, Crowe SE, Leary JF, Gourley WK, Luthra GK, Brooks EG, Graham DY, Reyes VE and Ernst PB (1998). "Lymphocytes in the human gastric mucosa during *Helicobacter pylori* have a T helper cell 1 phenotype." Gastroenterology 114(3): 482-92.

Beil W, Obst B, Sewing KF and Wagner S (2000). "*Helicobacter pylori* reduces intracellular glutathione in gastric epithelial cells." Dig Dis Sci 45(9): 1769-73.

Benaissa M, Babin P, Quellard N, Pezennec L, Cenatiempo Y and Fauchere JL (1996). "Changes in *Helicobacter pylori* ultrastructure and antigens during conversion from the bacillary to the coccoid form." Infect Immun 64(6): 2331-5.

Bennedsen M, Wang X, Willen R, Wadstrom T and Andersen LP (1999). "Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes." Immunol Lett 70(3): 185-9. **[Paper VI]**

Berdanier CD and Everts HB (2001). "Mitochondrial DNA in aging and degenerative disease." Mutat Res 475(1-2): 169-83.

Berg DJ, Lynch NA, Lynch RG and Lauricella DM (1998). "Rapid development of severe hyperplastic gastritis with gastric epithelial dedifferentiation in *Helicobacter felis*-infected IL-10(-/-) mice." Am J Pathol 152(5): 1377-86.

Blaser MJ and Berg DE (2001). "*Helicobacter pylori* genetic diversity and risk of human disease." J Clin Invest 107(7): 767-73.

Bode G, Mauch F and Malfertheiner P (1993). "The coccoid forms of *Helicobacter pylori*. Criteria for their viability." Epidemiol Infect 111(3): 483-90.

Bode G, Rothenbacher D, Brenner H and Adler G (1998). "Pets are not a risk factor for *Helicobacter pylori* infection in young children: results of a population-based study in Southern Germany." Pediatr Infect Dis J 17(10): 909-12.

Bouzourene H, Haefliger T, Delacretaz F and Saraga E (1999). "The role of *Helicobacter pylori* in primary gastric MALT lymphoma." Histopathology 34(2): 118-23.

Boyanova L and Neshev G (1999). "Inhibitory effect of rose oil products on *Helicobacter pylori* growth in vitro: preliminary report." J Med Microbiol 48(7): 705-6.

Buiatti E, Palli D, Decarli A, Amadori D, Avellini C, Bianchi S, Bonaguri C, Cipriani F, Cocco P, Giacosa A and et al. (1990). "A case-control study of gastric cancer and diet in Italy: II. Association with nutrients." Int J Cancer 45(5): 896-901.

Cantorna MT and Balish E (1990). "Inability of human clinical strains of *Helicobacter pylori* to colonize the alimentary tract of germfree rodents." Can J Microbiol 36(4): 237-41.

Cavalca V, Cighetti G, Bamonti F, Loaldi A, Bortone L, Novembrino C, De Franceschi M, Belardinelli R and Guazzi MD (2001). "Oxidative Stress and Homocysteine in Coronary Artery Disease." Clin Chem 47(5): 887-892.

Cave DR (1997). "How is *Helicobacter pylori* transmitted?" Gastroenterology 113(6 Suppl): S9-14.

Cellini L, Allocati N, Angelucci D, Iezzi T, Di Campli E, Marzio L and Dainelli B (1994). "Coccoid *Helicobacter pylori* not culturable in vitro reverts in mice." Microbiol Immunol 38(11): 843-50.

Cellini L, Di Campli E, Masulli M, Di Bartolomeo S and Allocati N (1996). "Inhibition of *Helicobacter pylori* by garlic extract (Allium sativum)." FEMS Immunol Med Microbiol 13(4): 273-7.

Cellini L, Robuffo I, Maraldi NM and Donelli G (2001). "Searching the point of no return in *Helicobacter pylori* life: necrosis and/or programmed death?" J Appl Microbiol 90(5): 727-32.

Chan JK, Ng CS and Isaacson PG (1990). "Relationship between high-grade lymphoma and low-grade B-cell mucosa-associated lymphoid tissue lymphoma (MALToma) of the stomach." Am J Pathol 136(5): 1153-64.

Chan WY, Hui PK, Leung KM, Chow J, Kwok F and Ng CS (1994). "Coccoid forms of *Helicobacter pylori* in the human stomach." Am J Clin Pathol 102(4): 503-7.

Chen D, Friis-Hansen L, Wang X, Zhao CM, Waldum H, Halgunset J and Wadström T (2001). "Different effects of chronic *Helicobacter pylori* infection on parietal and ECL cells between wild type and gastrin-deficient mice." GASTROENTEROLOGY 120 Suppl. 1(5): A728.

Chen W, Shu D and Chadwick VS (1999). "*Helicobacter pylori* infection in interleukin-4-deficient and transgenic mice." Scand J Gastroenterol 34(10): 987-92.

Chew BP (1993). "Role of carotenoids in the immune response." J Dairy Sci 76(9): 2804-11.

Chew BP, Park JS, Wong MW and Wong TS (1999). "A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice in vivo." Anticancer Res 19(3A): 1849-53.

Cohen M and Bhagavan HN (1995). "Ascorbic acid and gastrointestinal cancer." J Am Coll Nutr 14(6): 565-78.

Correa P (1992a). "Diet modification and gastric cancer prevention." J Natl Cancer Inst Monogr 12: 75-8.

Correa P (1992b). "Human gastric carcinogenesis: a multistep and multifactorial process-- First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention." Cancer Res 52(24): 6735-40.

Correa P, Malcom G, Schmidt B, Fontham E, Ruiz B, Bravo JC, Bravo LE, Zarama G and Realpe JL (1998). "Review article: Antioxidant micronutrients and gastric cancer." Aliment Pharmacol Ther 12 Suppl 1: 73-82.

Crabtree JE (1998). "Eradication of chronic *Helicobacter pylori* infection by therapeutic vaccination." Gut 43(1): 7-8.

Crabtree JE, Covacci A, Farmery SM, Xiang Z, Tompkins DS, Perry S, Lindley IJ and Rappuoli R (1995). "*Helicobacter pylori* induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype." J Clin Pathol 48(1): 41-5.

Crowe SE, Alvarez L, Dytoc M, Hunt RH, Muller M, Sherman P, Patel J, Jin Y and Ernst PB (1995). "Expression of interleukin 8 and CD54 by human gastric epithelium after *Helicobacter pylori* infection in vitro." Gastroenterology 108(1): 65-74.

Czinn SJ and Nedrud JG (1997). "Immunopathology of *Helicobacter pylori* infection and disease." Springer Semin Immunopathol 18(4): 495-513.

Dajani EZ and Klamut MJ (2000). "Novel therapeutic approaches to gastric and duodenal ulcers: an update." Expert Opin Investig Drugs 9(7): 1537-44.

Danesh J, Lawrence M, Murphy M, Roberts S and Collins R (2000a). "Systematic review of the epidemiological evidence on *Helicobacter pylori* infection and nonulcer or uninvestigated dyspepsia." Arch Intern Med 160(8): 1192-8.

Danesh J and Pounder RE (2000b). "Eradication of *Helicobacter pylori* and non-ulcer dyspepsia." Lancet 355(9206): 766-7.

Danielsson D and Jurstrand M (1998). "Nonopsonic activation of neutrophils by *Helicobacter pylori* is inhibited by rebamipide." Dig Dis Sci 43(9 Suppl): 167S-173S.

Danon SJ and Eaton KA (1998a). "The role of gastric *Helicobacter* and N-methyl-N'-nitro- N- nitrosoguanidine in carcinogenesis of mice." Helicobacter 3(4): 260-8.

Danon SJ, Luria BJ, Mankoski RE and Eaton KA (1998b). "RFLP and RAPD analysis of *in vivo* genetic interactions between strains of *Helicobacter pylori*." Helicobacter 3(4): 254-9.

Daroch F, Hoeneisen M, Gonzalez CL, Kawaguchi F, Salgado F, Solar H and Garcia A (2001). "In vitro antibacterial activity of Chilean red wines against *Helicobacter pylori*." Microbios 104(408): 79-85.

De Wolf-Peeters C and Achten R (1999). "The histogenesis of large-cell gastric lymphomas." Histopathology 34(1): 71-5.

Del Prete G (1998). "The concept of type-1 and type-2 helper T cells and their cytokines in humans." Int Rev Immunol 16(3-4): 427-55.

D'Elios MM, Manghetti M, De Carli M, Costa F, Baldari CT, Burroni D, Telford JL, Romagnani S and Del Prete G (1997). "T helper 1 effector cells specific for *Helicobacter pylori* in the gastric antrum of patients with peptic ulcer disease." J Immunol 158(2): 962-7.

Desai HG, Gill HH, Shankaran K, Mehta PR and Prabhu SR (1991). "Dental plaque: a permanent reservoir of *Helicobacter pylori*?" Scand J Gastroenterol 26(11): 1205-8.

Dieterich C, Bouzourene H, Blum AL and Corthesy-Theulaz IE (1999). "Urease-based mucosal immunization against *Helicobacter heilmannii* infection induces corpus atrophy in mice." Infect Immun 67(11): 6206-9.

Dixon MF, Genta RM, Yardley JH and Correa P (1996). "Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994." Am J Surg Pathol 20(10): 1161-81.

Donelli G, Matarrese P, Fiorentini C, Dainelli B, Taraborelli T, Di Campli E, Di Bartolomeo S and Cellini L (1998). "The effect of oxygen on the growth and cell morphology of *Helicobacter pylori*." FEMS Microbiol Lett 168(1): 9-15.

Dore MP, Bilotta M, Vaira D, Manca A, Massarelli G, Leandro G, Atzei A, Pisanu G, Graham DY and Realdi G (1999a). "High prevalence of *Helicobacter pylori* infection in shepherds." Dig Dis Sci 44(6): 1161-4.

Dore MP, Sepulveda AR, Osato MS, Realdi G and Graham DY (1999b). "*Helicobacter pylori* in sheep milk." Lancet 354(9173): 132.

Drago L, Mombelli B, Ciardo G, De Vecchi E and Gismondo MR (1999). "Effects of three different fish oil formulations on *Helicobacter pylori* growth and viability: in vitro study." J Chemother 11(3): 207-10.

Drake IM, Davies MJ, Mapstone NP, Dixon MF, Schorah CJ, White KL, Chalmers DM and Axon AT (1996). "Ascorbic acid may protect against human gastric cancer by scavenging mucosal oxygen radicals." Carcinogenesis 17(3): 559-62.

Dubois A, Berg DE, Incecik ET, Fiala N, Heman-Ackah LM, Perez-Perez GI and Blaser MJ (1996). "Transient and persistent experimental infection of nonhuman primates with *Helicobacter pylori*: implications for human disease." Infect Immun 64(8): 2885-91.

Dubois A, Fiala N, Heman-Ackah LM, Drazek ES, Tarnawski A, Fishbein WN, Perez-Perez GI and Blaser MJ (1994). "Natural gastric infection with *Helicobacter pylori* in monkeys: a model for spiral bacteria infection in humans." Gastroenterology 106(6): 1405-17.

Dubois A, Lee CK, Fiala N, Kleanthous H, Mehlman PT and Monath T (1998). "Immunization against natural *Helicobacter pylori* infection in nonhuman primates." Infect Immun 66(9): 4340-6.

Eaton KA, Catrenich CE, Makin KM and Krakowka S (1995). "Virulence of coccoid and bacillary forms of *Helicobacter pylori* in gnotobiotic piglets." J Infect Dis 171(2): 459-62.

Eaton KA, Kersulyte D, Mefford M, Danon SJ, Krakowka S and Berg DE (2001). "Role of *Helicobacter pylori* cag region genes in colonization and gastritis in two animal models." Infect Immun 69(5): 2902-8.

Eaton KA, Radin MJ and Krakowka S (1993). "Animal models of bacterial gastritis: the role of host, bacterial species and duration of infection on severity of gastritis." Zentralbl Bakteriol 280(1-2): 28-37.

Eaton KA, Suerbaum S, Josenhans C and Krakowka S (1996). "Colonization of gnotobiotic piglets by *Helicobacter pylori* deficient in two flagellin genes." Infect Immun 64(7): 2445-8.

Eidt S and Stolte M (1995). "The significance of *Helicobacter pylori* in relation to gastric cancer and lymphoma." Eur J Gastroenterol Hepatol 7(4): 318-21.

Emerit I, Serejo F, Filipe P, Alaoui Youssefi A, Fernandes A, Costa A, Freitas J, Ramalho F, Baptista A and Carneiro de Moura M (2000). "Clastogenic factors as biomarkers of oxidative stress in chronic hepatitis C." Digestion 62(2-3): 200-7.

Engstrand L, Gustavsson S, Jorgensen A, Schwan A and Scheynius A (1990). "Inoculation of barrier-born pigs with *Helicobacter pylori*: a useful animal model for gastritis type B." Infect Immun 58(6): 1763-8.

Enno A, O'Rourke J, Braye S, Howlett R and Lee A (1998). "Antigen-dependent progression of mucosa-associated lymphoid tissue (MALT)-type lymphoma in the stomach. Effects of antimicrobial therapy on gastric MALT lymphoma in mice." Am J Pathol 152(6): 1625-32.

Enno A, O'Rourke JL, Howlett CR, Jack A, Dixon MF and Lee A (1995). "MALToma-like lesions in the murine gastric mucosa after long-term infection with *Helicobacter felis*. A mouse model of *Helicobacter pylori*- induced gastric lymphoma." Am J Pathol 147(1): 217-22.

Enroth H and Engstrand L (1996). "Egg passage of rodshaped and coccoid forms of *Helicobacter pylori*: preliminary studies." Helicobacter 1(3): 183-6.

Enroth H and Engstrand L (2000). "[*Helicobacter pylori*--the discovery still of current interest in the new millenium. Both good and bad bacteria in the stomach?]." Lakartidningen 97(3): 150-3.

Erdman SE, Correa P, Coleman LA, Schrenzel MD, Li X and Fox JG (1997). "*Helicobacter mustelae*-associated gastric MALT lymphoma in ferrets." Am J Pathol 151(1): 273-80.

Ermak TH, Giannasca PJ, Nichols R, Myers GA, Nedrud J, Weltzin R, Lee CK, Kleanthous H and Monath TP (1998). "Immunization of mice with urease vaccine affords protection against *Helicobacter pylori* infection in the absence of antibodies and is mediated by MHC class II-restricted responses." J Exp Med 188(12): 2277-88.

Ernst P (1999). "Review article: the role of inflammation in the pathogenesis of gastric cancer." Aliment Pharmacol Ther 13 Suppl 1: 13-8.

Ernst PB and Pappo J (2000). "Preventive and therapeutic vaccines against *Helicobacter pylori*: current status and future challenges." Curr Pharm Des 6(15): 1557-73.

Esteves MI, Schrenzel MD, Marini RP, Taylor NS, Xu S, Hagen S, Feng Y, Shen Z and Fox JG (2000). "*Helicobacter pylori* gastritis in cats with long-term natural infection as a model of human disease." Am J Pathol 156(2): 709-21.

Evans DJ, Jr. and Evans DG (2000). "*Helicobacter pylori* adhesins: review and perspectives." Helicobacter 5(4): 183-95.

Evans WJ (2000). "Vitamin E, vitamin C, and exercise." Am J Clin Nutr 72(2 Suppl): 647S-52S.

Falk PG, J. SA, Guruge JL, Engstrand LG and Gordon JI (2001). Transgenic mouse medels for studying the relationship between *Helicobacter pylori* infection and gastric cancer. *Helicobacter pylori*: Molecular and Cellular Biology. Achtman M and Suerbaum S. Wymondham, Horizon Scientific Press: 133-153.

Falk PG, Syder AJ, Guruge JL, Kirschner D, Blaser MJ and Gordon JI (2000). "Theoretical and experimental approaches for studying factors defining the *Helicobacter pylori*-host relationship." Trends Microbiol 8(7): 321-9.

Fan L, Vonshak A, Zarka A and Boussiba S (1998). "Does astaxanthin prot*ect Haematococcus* against light damage?" Z Naturforsch 53c: 93-100.

Faure H, Fayol V, Galabert C, Grolier P, Moel GL, Stephens J and Nabet F (1999). "[Carotenoids: 2. Diseases and supplementation studies]." Ann Biol Clin (Paris) 57(3): 273-82.

Ferrero RL, Ave P, Radcliff FJ, Labigne A and Huerre MR (2000). "Outbred mice with long-term *Helicobacter felis* infection develop both gastric lymphoid tissue and glandular hyperplastic lesions." J Pathol 191(3): 333-40.

Ferrero RL and Fox JG (2001a). In vivo modeling of *Helicobacter*-associated gastrointestinal diseases. *Helicobacter pylori*: Physiology and Genetics. Mobley HLT, Mendz GL and Hazell SL. Washington, D.C., ASM press: 565-582.

Ferrero RL and Jenks PJ (2001b). In vivo adaptation to the host. *Helicobacter pylori*: Physiology and Genetics. Mobley HLT, Mendz GL and Hazell SL. *Washington, D.C.*, ASM press: 583-592.

Ferrero RL, Thiberge JM, Huerre M and Labigne A (1998). "Immune responses of specific-pathogen-free mice to chronic *Helicobacter pylori* (strain SS1) infection." Infect Immun 66(4): 1349-55.

Ferrero RL, Thiberge JM, Kansau I, Wuscher N, Huerre M and Labigne A (1995). "The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice." Proc Natl Acad Sci U S A 92(14): 6499-503.

Foss HD, Schmitt-Graff A, Daum S, Anagnostopoulos I, Assaf C, Hummel M and Stein H (1999). "Origin of primary gastric T-cell lymphomas from intraepithelial T- lymphocytes: report of two cases." Histopathology 34(1): 9-15.

Fox JG, Beck P, Dangler CA, Whary MT, Wang TC, Shi HN and Nagler-Anderson C (2000). "Concurrent enteric *helminth* infection modulates inflammation and gastric immune responses and reduces *Helicobacter*-induced gastric atrophy." Nat Med 6(5): 536-42.

Fox JG, Cabot EB, Taylor NS and Laraway R (1988). "Gastric colonization by *Campylobacter pylori* subsp. *mustelae* in ferrets." Infect Immun 56(11): 2994-6.

Fox JG, Correa P, Taylor NS, Lee A, Otto G, Murphy JC and Rose R (1990). "*Helicobacter mustelae*-associated gastritis in ferrets. An animal model of *Helicobacter pylori* gastritis in humans." Gastroenterology 99(2): 352-61.

Fox JG and Lee A (1997). "The role of *Helicobacter* species in newly recognized gastrointestinal tract diseases of animals." Lab Anim Sci 47(3): 222-55.

Fox JG, Lee A, Otto G, Taylor NS and Murphy JC (1991a). "*Helicobacter felis* gastritis in gnotobiotic rats: an animal model of *Helicobacter pylori* gastritis." Infect Immun 59(3): 785-91.

Fox JG, Otto G, Murphy JC, Taylor NS and Lee A (1991b). "Gastric colonization of the ferret with *Helicobacter* species: natural and experimental infections." Rev Infect Dis 13 Suppl 8: S671-80.

Fox JG, Wishnok JS, Murphy JC, Tannenbaum SR and Correa P (1993). "MNNG-induced gastric carcinoma in ferrets infected with *Helicobacter mustelae*." Carcinogenesis 14(9): 1957-61.

Fukuda Y, Yamamoto I, Okui M, Tonokatsu Y, Tamura K and Shimoyama T (1995). "Combination therapy with mucosal protective agent for the eradication of *Helicobacter pylori*." Eur J Gastroenterol Hepatol 7 Suppl 1: S45-7.

Ghiara P, Rossi M, Marchetti M, Di Tommaso A, Vindigni C, Ciampolini F, Covacci A, Telford JL, De Magistris MT, Pizza M, Rappuoli R and Del Giudice G (1997). "Therapeutic intragastric vaccination against *Helicobacter pylori* in

mice eradicates an otherwise chronic infection and confers protection against reinfection." Infect Immun 65(12): 4996-5002.

Gomez-Duarte OG, Bumann D and Meyer TF (1999). "The attenuated Salmonella vaccine approach for the control of *Helicobacter pylori*-related diseases." Vaccine 17(13-14): 1667-73.

Goodman KJ, Correa P, Tengana Aux HJ, DeLany JP and Collazos T (1997). "Nutritional factors and *Helicobacter pylori* infection in Colombian children." J Pediatr Gastroenterol Nutr 25(5): 507-15.

Goodwin CS, Armstrong JA, Chilvers T, Peters M, Collins MD, Sly L, McConnell W and Harper WES (1989). "Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively." Int J Syst Bacteriol 39: 397-405.

Grisham MB, Jourd'heuil D and Wink DA (2000). "Review article: chronic inflammation and reactive oxygen and nitrogen metabolism--implications in DNA damage and mutagenesis." Aliment Pharmacol Ther 14 Suppl 1: 3-9.

Grubel P, Huang L, Masubuchi N, Stutzenberger FJ and Cave DR (1998). "Detection of *Helicobacter pylori* DNA in houseflies (Musca domestica) on three continents." Lancet 352(9130): 788-9.

Guruge JL, Falk PG, Lorenz RG, Dans M, Wirth HP, Blaser MJ, Berg DE and Gordon JI (1998). "Epithelial attachment alters the outcome of *Helicobacter pylori* infection." Proc Natl Acad Sci U S A 95(7): 3925-30.

Gutteridge JM and Halliwell B (2000). "Free radicals and antioxidants in the year 2000. A historical look to the future." Ann N Y Acad Sci 899: 136-47.

Hachem CY, Clarridge JE, Evans DG and Graham DY (1995). "Comparison of agar based media for primary isolation of *Helicobacter pylori*." J Clin Pathol 48(8): 714-6.

Haeberle HA, Kubin M, Bamford KB, Garofalo R, Graham DY, El-Zaatari F, Karttunen R, Crowe SE, Reyes VE and Ernst PB (1997). "Differential stimulation of interleukin-12 (IL-12) and IL-10 by live and killed *Helicobacter pylori in vitro* and association of IL-12 production with gamma interferon-producing T cells in the human gastric mucosa." Infect Immun 65(10): 4229-35.

Hahm KB, Lee KJ, Kim JH, Cho SW and Chung MH (1998). "*Helicobacter pylori* infection, oxidative DNA damage, gastric carcinogenesis, and reversibility by rebamipide." Dig Dis Sci 43(9 Suppl): 72S-77S.

Handt LK, Fox JG, Dewhirst FE, Fraser GJ, Paster BJ, Yan LL, Rozmiarek H, Rufo R and Stalis IH (1994). "*Helicobacter pylori* isolated from the domestic cat: public health implications." Infect Immun 62(6): 2367-74.

Hansson LE, Nyren O, Hsing AW, Bergstrom R, Josefsson S, Chow WH, Fraumeni JF, Jr. and Adami HO (1996). "The risk of stomach cancer in patients with gastric or duodenal ulcer disease." N Engl J Med 335(4): 242-9.

Hayashi S, Sugiyama T, Amano K, Isogai H, Isogai E, Aihara M, Kikuchi M, Asaka M, Yokota K, Oguma K, Fujii N and Hirai Y (1998). "Effect of rebamipide, a novel antiulcer agent, on *Helicobacter pylori* adhesion to gastric epithelial cells." Antimicrob Agents Chemother 42(8): 1895-9.

Hocking D, Webb E, Radcliff F, Rothel L, Taylor S, Pinczower G, Kapouleas C, Braley H, Lee A and Doidge C (1999). "Isolation of recombinant protective *Helicobacter pylori* antigens." Infect Immun 67(9): 4713-9.

Hojo M, Miwa H, Kikuchi S and Sato N (2000). "Do mucosal defensive agents improve the cure rate when used with dual or triple therapy regimens for eradicating *Helicobacter pylori* infection?" Aliment Pharmacol Ther 14(2): 193-201.

Honda S, Fujioka T, Tokieda M, Satoh R, Nishizono A and Nasu M (1998). "Development of *Helicobacter pylori*-induced gastric carcinoma in Mongolian gerbils." Cancer Res 58(19): 4255-9.

Hsi ED, Eisbruch A, Greenson JK, Singleton TP, Ross CW and Schnitzer B (1998). "Classification of primary gastric lymphomas according to histologic features." Am J Surg Pathol 22(1): 17-27.

Hussell T, Isaacson PG, Crabtree JE and Spencer J (1993). "The response of cells from low-grade B-cell gastric lymphomas of mucosa- associated lymphoid tissue to *Helicobacter pylori* [see comments]." Lancet 342(8871): 571-4.

Hussell T, Isaacson PG, Crabtree JE and Spencer J (1996). "*Helicobacter pylori*-specific tumour-infiltrating T cells provide contact dependent help for the growth of malignant B cells in low-grade gastric lymphoma of mucosa-associated lymphoid tissue." J Pathol 178(2): 122-7.

Iinuma S, Naito Y, Yoshikawa T, Takahashi S, Takemura T, Yoshida N and Kondo M (1998). "*In vitro* studies indicating antioxidative properties of rebamipide." Dig Dis Sci 43(9 Suppl): 35S-39S.

Ikeno T, Ota H, Sugiyama A, Ishida K, Katsuyama T, Genta RM and Kawasaki S (1999). "*Helicobacter pylori*-induced chronic active gastritis, intestinal metaplasia, and gastric ulcer in Mongolian gerbils." Am J Pathol 154(3): 951-60.

Inoue M, Nishikawa M, Sato EF, Ah-Mee P, Kashiba M, Takehara Y and Utsumi K (1999). "Cross-talk of NO, superoxide and molecular oxygen, a majesty of aerobic life." Free Radic Res 31(4): 251-60.

Isaacson PG (1994). "Gastrointestinal lymphoma." Hum Pathol 25(10): 1020-9.

Isaacson PG (1996). "Recent developments in our understanding of gastric lymphomas." Am J Surg Pathol 20(Suppl 1): S1-7.

Isaacson PG (1999). "Gastrointestinal lymphomas of T- and B-cell types." Mod Pathol 12(2): 151-8.

Jaakkimainen RL, Boyle E and Tudiver F (1999). "Is *Helicobacter pylori* associated with non-ulcer dyspepsia and will eradication improve symptoms? A meta-analysis." Bmj 319(7216): 1040-4.

Jarosz M, Dzieniszewski J, Dabrowska-Ufniarz E, Wartanowicz M and Ziemlanski S (2000). "Tobacco smoking and vitamin C concentration in gastric juice in healthy subjects and patients with *Helicobacter pylori* infection." Eur J Cancer Prev 9(6): 423-8.

Jarosz M, Dzieniszewski J, Dabrowska-Ufniarz E, Wartanowicz M, Ziemlanski S and Reed PI (1998). "Effects of high dose vitamin C treatment on *Helicobacter pylori* infection and total vitamin C concentration in gastric juice." Eur J Cancer Prev 7(6): 449-54.

Jones NL, Shabib S and Sherman PM (1997). "Capsaicin as an inhibitor of the growth of the gastric pathogen *Helicobacter pylori*." FEMS Microbiol Lett 146(2): 223-7.

Jonkers D, Gisbertz I, de Bruine A, Bot F, Arends JW, Stobberingh E, Schouten H and Stockbrugger R (1997). "*Helicobacter pylori* and non-*Helicobacter pylori* bacterial flora in gastric mucosal and tumour specimens of patients with primary gastric lymphoma." Eur J Clin Invest 27(11): 885-92.

Jyonouchi H, Sun S, Mizokami M and Gross MD (1996). "Effects of various carotenoids on cloned, effector-stage T-helper cell activity." Nutr Cancer 26(3): 313-24.

Jyonouchi H, Sun S, Tomita Y and Gross MD (1995). "Astaxanthin, a carotenoid without vitamin A activity, augments antibody responses in cultures including T-helper cell clones and suboptimal doses of antigen." J Nutr 125(10): 2483-92.

Kanke Y, Iitoi Y, Iwasaki M, Iwase Y, Iwama M, Kimira M, Takahashi T, Tsugane S, Watanabe S and Akabane M (1996). "Effects of human diets of two different Japanese populations on cancer incidence in rat hepatic drug-metabolizing and antioxidant enzyme systems." Nutr Cancer 26(1): 63-71.

Karita M, Kouchiyama T, Okita K and Nakazawa T (1991). "New small animal model for human gastric *Helicobacter pylori* infection: success in both nude and euthymic mice." Am J Gastroenterol 86(11): 1596-603.

Kashimura H, Suzuki K, Hassan M, Ikezawa K, Sawahata T, Watanabe T, Nakahara A, Mutoh H and Tanaka N (1999). "Polaprezinc, a mucosal protective agent, in combination with lansoprazole, amoxycillin and clarithromycin increases the cure rate of *Helicobacter pylori* infection." Aliment Pharmacol Ther 13(4): 483-7.

Kaufmann SHE and Kabelitz D (1998). Methods in Microbiology, Immunology of infection. San Diego, Academic press.

Keates S, Hitti YS, Upton M and Kelly CP (1997). "*Helicobacter pylori* infection activates NF-kappa B in gastric epithelial cells." Gastroenterology 113(4): 1099-109.

Kemp M, Theander TG and Kharazmi A (1996). "The contrasting roles of CD4+ T cells in intracellular infections in humans: leishmaniasis as an example." Immunol Today 17(1): 13-6.

Khaled MA and Sarker SA (1998). "Changes of oxidant and antioxidant status in humans due to *H. pylori* infection." Nutri Res 18: 1463-1468.

Kidd M and Modlin IM (1998). "A century of *Helicobacter pylori*: paradigms lost-paradigms regained." Digestion 59(1): 1-15.

Kim H, Seo JY and Kim KH (2000). "Inhibition of lipid peroxidation, NF-kappaB activation and IL-8 production by rebamipide in *Helicobacter pylori*-stimulated gastric epithelial cells." Dig Dis Sci 45(3): 621-8.

Koga T, Kawada H, Utsui Y, Domon H, Ishii C and Yasuda H (1996). "*In-vitro* and *in-vivo* antibacterial activity of plaunotol, a cytoprotective antiulcer agent, against *Helicobacter pylori*." J Antimicrob Chemother 37(5): 919-29.

Kong L, Smith JG, Bramhill D, Abruzzo GK, Bonfiglio C, Cioffe C, Flattery AM, Gill CJ, Lynch L, Scott PM, Silver L, Thompson C, Kropp H and Bartizal K (1996). "A sensitive and specific PCR method to detect *Helicobacter felis* in a conventional mouse model." Clin Diagn Lab Immunol 3(1): 73-8.

Kong Q, Beel JA and Lillehei KO (2000). "A threshold concept for cancer therapy." Med Hypotheses 55(1): 29-35.

Krakowka S, Morgan DR, Kraft WG and Leunk RD (1987). "Establishment of gastric *Campylobacter pylori* infection in the neonatal gnotobiotic piglet." Infect Immun 55(11): 2789-96.

Kuipers EJ (1999). "Review article: exploring the link between *Helicobacter pylori* and gastric cancer." Aliment Pharmacol Ther 13 Suppl 1: 3-11.

Kumagai T, Yan J, Graham DY, Tozuka M, Okimura Y, Ikeno T, Sugiyama A, Katsuyama T and Ota H (2001). "Serum Immunoglobulin G Immune Response to *Helicobacter pylori* Antigens in Mongolian Gerbils." J Clin Microbiol 39(4): 1283-8.

Kurashige M, Okimasu E, Inoue M and Utsumi K (1990). "Inhibition of oxidative injury of biological membranes by astaxanthin." Physiol Chem and Physics and Medical Nmr 22: 27-38.

Lahaie RG and Gaudreau C (2000). "*Helicobacter pylori* antibiotic resistance: trends over time." Can J Gastroenterol 14(10): 895-9.

Lambert JR, Borromeo M, Pinkard KJ, Turner H, Chapman CB and Smith ML (1987). "Colonization of gnotobiotic piglets with *Campylobacter Pyloridis*--an animal model?" J Infect Dis 155(6): 1344.

Lee A (1999). "Animal models of *Helicobacter* infection." Mol Med Today 5(11): 500-1.

Lee A, Chen M, Coltro N, O'Rourke J, Hazell S, Hu P and Li Y (1993). "Long term infection of the gastric mucosa with *Helicobacter* species does induce atrophic gastritis in an animal model of *Helicobacter pylori* infection." Zentralbl Bakteriol 280(1-2): 38-50.

Lee A, Fox JG, Otto G and Murphy J (1990). "A small animal model of human *Helicobacter pylori* active chronic gastritis." Gastroenterology 99(5): 1315-23.

Lee A, O'Rourke J, De Ungria MC, Robertson B, Daskalopoulos G and Dixon MF (1997). "A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain." Gastroenterology 112(4): 1386-97.

Lee CK (2001). "Vaccination against *Helicobacter pylori* in non-human primate models and humans." Scand J Immunol 53(5): 437-42.

Lelwala-Guruge J, Nilsson I, Ljungh A and Wadstrom T (1992). "Cell surface proteins of *Helicobacter pylori* as antigens in an ELISA and a comparison with three commercial ELISA." Scand J Infect Dis 24(4): 457-65.

Leung WK and Graham DY (2000). "Clarithromycin for *Helicobacter pylori* infection." Expert Opin Pharmacother 1(3): 507-14.

Levine M (1986). "New concepts in the biology and biochemistry of ascorbic acid." N Engl J Med 314(14): 892-902.

Li H, Andersson EM and Helander HF (1999). "Reactions from rat gastric mucosa during one year of *Helicobacter pylori* infection." Dig Dis Sci 44(1): 116-24.

Li H, Kalies I, Mellgard B and Helander HF (1998). "A rat model of chronic *Helicobacter pylori* infection. Studies of epithelial cell turnover and gastric ulcer healing." Scand J Gastroenterol 33(4): 370-8.

Li S, Taylor KB, Kelly SJ and Jedrzejas MJ (2001). "Vitamin C inhibits the enzymatic activity of Streptococcus pneumoniae hyaluronate lyase." J Biol Chem 276(18): 15125-30.

Lignell Å, Surace R, Böttiger P and Borody T (1999). Symptom improvement in *Helicobacter pylori*-positive non-ucler dyspeptic patients after treatment with carotenoid astaxanthin. The 12th International Carotenoid Symposium, Cairns, Australia.

Lindholm C, Quiding-Jarbrink M, Lonroth H, Hamlet A and Svennerholm AM (1998). "Local cytokine response in *Helicobacter pylori*-infected subjects." Infect Immun 66(12): 5964-71.

Luzza F, Mancuso M, Imeneo M, Contaldo A, Giancotti L, Pensabene L, Doldo P, Liberto MC, Strisciuglio P, Foca A, Guandalini S and Pallone F (2000). "Evidence favouring the gastro-oral route in the transmission of *Helicobacter pylori* infection in children." Eur J Gastroenterol Hepatol 12(6): 623-7.

Mabe K, Yamada M, Oguni I and Takahashi T (1999). "*In vitro* and *in vivo* activities of tea catechins against *Helicobacter pylori*." Antimicrob Agents Chemother 43(7): 1788-91.

Mahady GB and Pendland SL (2000). "Resveratrol inhibits the growth of *Helicobacter pylori in vitro*." Am J Gastroenterol 95(7): 1849.

Malaty HM, Evans DG, Evans DJ, Jr. and Graham DY (1992). "*Helicobacter pylori* in Hispanics: comparison with blacks and whites of similar age and socioeconomic class." Gastroenterology 103(3): 813-6.

Malaty HM and Graham DY (1994). "Importance of childhood socioeconomic status on the current prevalence of *Helicobacter pylori* infection." Gut 35(6): 742-5.

Mannick EE, Bravo LE, Zarama G, Realpe JL, Zhang XJ, Ruiz B, Fontham ET, Mera R, Miller MJ and Correa P (1996). "Inducible nitric oxide synthase, nitrotyrosine, and apoptosis in *Helicobacter pylori* gastritis: effect of antibiotics and antioxidants." Cancer Res 56(14): 3238-43.

Marchetti M, Arico B, Burroni D, Figura N, Rappuoli R and Ghiara P (1995). "Development of a mouse model of *Helicobacter pylori* infection that mimics human disease." Science 267(5204): 1655-8.

Marshall BJ (1994). "*Helicobacter pylori*." Am J Gastroenterol 89(8 Suppl): S116-28.

Marshall BJ, Armstrong JA, McGechie DB and Glancy RJ (1985). "Attempt to fulfil Koch's postulates for pyloric Campylobacter." Med J Aust 142(8): 436-9.

Marshall BJ and Warren JR (1984). "Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration." Lancet 1(8390): 1311-5.

Marshall E (2001). "Genome sequencing. Celera assembles mouse genome; public labs plan new strategy." Science 292(5518): 822.

Matolcsy A, Nagy M, Kisfaludy N and Kelenyi G (1999). "Distinct clonal origin of low-grade MALT-type and high-grade lesions of a multifocal gastric lymphoma." Histopathology 34(1): 6-8.

Matsumoto S, Washizuka Y, Matsumoto Y, Tawara S, Ikeda F, Yokota Y and Karita M (1997). "Induction of ulceration and severe gastritis in Mongolian gerbil by *Helicobacter pylori* infection." J Med Microbiol 46(5): 391-7.

Mayo K, Castagnino C and Chèze C (2000). "Amelioration of gastric inflammation by polyphenols from red wine: a mouse model using *Helicobacter felis*." Gut supp 1 47: A61(8/11).

McCarthy DM (1998). "*H. pylori* infection in non-ulcer patients--to treat or not to treat. The case against treatment." Eur J Surg Suppl 582: 11-5.

McCord JM (2000). "The evolution of free radicals and oxidative stress." Am J Med 108(8): 652-9.

McNulty CA, Dent JC, Curry A, Uff JS, Ford GA, Gear MW and Wilkinson SP (1989). "New spiral bacterium in gastric mucosa." J Clin Pathol 42(6): 585-91.

Mendall MA, Goggin PM, Molineaux N, Levy J, Toosy T, Strachan D and Northfield TC (1992). "Childhood living conditions and *Helicobacter pylori* seropositivity in adult life." Lancet 339(8798): 896-7.

Miki W (1991). "Biological functions and activities of animal carotenoids." Pure Appl Chem 63(1): 141-6.

Misiewicz JJ (1991). "The Sydney System: a new classification of gastritis. Introduction." J Gastroenterol Hepatol 6(3): 207-8.

Mitchell HM, Li YY, Hu PJ, Liu Q, Chen M, Du GG, Wang ZJ, Lee A and Hazell SL (1992). "Epidemiology of *Helicobacter pylori* in southern China: identification of early childhood as the critical period for acquisition." J Infect Dis 166(1): 149-53.

Mizoguchi H, Fujioka T and Nasu M (1999). "Evidence for viability of coccoid forms of *Helicobacter pylori*." J Gastroenterol 34(Suppl 11): 32-6.

Moayyedi P, Soo S, Deeks J, Forman D, Mason J, Innes M and Delaney B (2000). "Systematic review and economic evaluation of *Helicobacter pylori* eradication treatment for non-ulcer dyspepsia. Dyspepsia Review Group." Bmj 321(7262): 659-64.

Mohammadi M, Nedrud J, Redline R, Lycke N and Czinn SJ (1997). "Murine CD4 T-cell response to *Helicobacter* infection: TH1 cells enhance gastritis and TH2 cells reduce bacterial load." Gastroenterology 113(6): 1848-57.

Mohammadi M, Redline R, Nedrud J and Czinn S (1996). "Role of the host in pathogenesis of *Helicobacter*-associated gastritis: *H. felis* infection of inbred and congenic mouse strains." Infect Immun 64(1): 238-45.

Moller H, Heseltine E and Vainio H (1995). "Working group report on schistosomes, liver flukes and *Helicobacter pylori*." Int J Cancer 60(5): 587-9.

Morgner A, Bayerdorffer E, Neubauer A and Stolte M (2000a). "Gastric MALT lymphoma and its relationship to *Helicobacter pylori* infection: Management and pathogenesis of the disease." Microsc Res Tech 48(6): 349-356.

Morgner A, Lehn N, Andersen LP, Thiede C, Bennedsen M, Trebesius K, Neubauer B, Neubauer A, Stolte M and Bayerdorffer E (2000b). "*Helicobacter heilmannii*-associated primary gastric low-grade MALT lymphoma: complete remission after curing the infection." Gastroenterology 118(5): 821-8.

Morgner A, Miehlke S, Fischbach W, Schmitt W, Muller-Hermelink H, Greiner A, Thiede C, Schetelig J, Neubauer A, Stolte M, Ehninger G and Bayerdorffer E (2001). "Complete Remission of Primary High-Grade B-Cell Gastric Lymphoma After Cure of *Helicobacter pylori* Infection." J Clin Oncol 19(7): 2041-8.

Murakami K, Fujioka T, Okimoto T, Mitsuishi Y, Oda T, Nishizono A and Nasu M (1999). "Analysis of p53 gene mutations in *Helicobacter pylori*-associated gastritis mucosa in endoscopic biopsy specimens." Scand J Gastroenterol 34(5): 474-7.

Mysore JV, Wigginton T, Simon PM, Zopf D, Heman-Ackah LM and Dubois A (1999). "Treatment of *Helicobacter pylori* infection in rhesus monkeys using a novel antiadhesion compound." Gastroenterology 117(6): 1316-25.

Naguib YM (2000). "Antioxidant activities of astaxanthin and related carotenoids." J Agric Food Chem 48(4): 1150-4.

Nakajima S, Graham DY, Hattori T and Bamba T (2000). "Strategy for treatment of *Helicobacter pylori* infection in adults. II. Practical policy in 2000." Curr Pharm Des 6(15): 1515-29.

Nakamura A, Park A, Nagata K, Sato EF, Kashiba M, Tamura T and Inoue M (2000). "Oxidative cellular damage associated with transformation of *Helicobacter pylori* from a bacillary to a coccoid form." Free Radic Biol Med 28(11): 1611-8.

Nakamura S, Aoyagi K, Furuse M, Suekane H, Matsumoto T, Yao T, Sakai Y, Fuchigami T, Yamamoto I, Tsuneyoshi M and Fujishima M (1998). "B-cell monoclonality precedes the development of gastric MALT lymphoma in *Helicobacter pylori*-associated chronic gastritis." Am J Pathol 152(5): 1271-9.

Nebiki H, Higuchi K, Arakawa T, Ando K, Uchida T, Ito H, Harihara S, Kuroki T and Kobayashi K (1998). "Effect of rebamipide on *Helicobacter pylori* infection in patients with peptic ulcer." Dig Dis Sci 43(9 Suppl): 203S-206S.

Neiger R and Simpson KW (2000). "*Helicobacte*r infection in dogs and cats: facts and fiction." J Vet Intern Med 14(2): 125-33.

Niki E, Noguchi N, Tsuchihashi H and Gotoh N (1995). "Interaction among vitamin C, vitamin E, and beta-carotene." Am J Clin Nutr 62(6 Suppl): 1322S-1326S.

Nilsson HO, Aleljung P, Nilsson I, Tyszkiewicz T and Wadström T (1996). "Immunomagnetic bead enrichment and PCR for detection of *Helicobacter pylori* in human stools." FEMS Immuno Med Microbiol 27: 73-79.

Nilsson I, Ljungh A, Aleljung P and Wadstrom T (1997). "Immunoblot assay for serodiagnosis of *Helicobacter pylori* infections." J Clin Microbiol 35(2): 427-32.

Nilsson I, Utt M, Nilsson HO, Ljungh A and Wadstrom T (2000). "Two-dimensional electrophoretic and immunoblot analysis of cell surface proteins of spiral-shaped and coccoid forms of *Helicobacter pylori* [In Process Citation]." Electrophoresis 21(13): 2670-7.

Noguchi N, Watanabe A and Shi H (2000). "Diverse functions of antioxidants." Free Radic Res 33(6): 809-17.

Nomura A, Stemmermann GN, Chyou PH, Kato I, Perez-Perez GI and Blaser MJ (1991). "*Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii." N Engl J Med 325(16): 1132-6.

Noroozi M, Angerson WJ and Lean ME (1998). "Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes." Am J Clin Nutr 67(6): 1210-8.

O'Gara EA, Hill DJ and Maslin DJ (2000). "Activities of garlic oil, garlic powder, and their diallyl constituents against *Helicobacter pylori*." Appl Environ Microbiol 66(5): 2269-73.

Ohkusa T, Takashimizu I, Fujiki K, Araki A, Ariake K, Shimoi K, Honda K, Enomoto Y, Sakurazawa T, Horiuchi T, Suzuki S, Ishii K and Ishikura T (1998). "Prospective evaluation of a new anti-ulcer agent, ecabet sodium, for the treatment of *Helicobacter pylori* infection." Aliment Pharmacol Ther 12(5): 457-61.

Ohta R, Yamada N, Kaneko H, Ishikawa K, Fukuda H, Fujino T and Suzuki A (1999). "In vitro inhibition of the growth of *Helicobacter pylori* by oil- macerated garlic constituents [letter]." Antimicrob Agents Chemother 43(7): 1811-2.

Okai Y and Higashi-Okai K (1996). "Possible immunomodulating activities of carotenoids in in vitro cell culture experiments." Int J Immunopharmacol 18(12): 753-8.

Oliver JD (1995). "The viable but non-culturable state in the human pathogen *Vibrio vulnificus*." FEMS Microbiol Lett 133: 203-8.

Opekun AR, El-Zaimaity HM, Osato MS, Gilger MA, Malaty HM, Terry M, Headon DR and Graham DY (1999). "Novel therapies for *Helicobacter pylori* infection." Aliment Pharmacol Ther 13(1): 35-42.

Osato MS, Ayub K, Le HH, Reddy R and Graham DY (1998). "Houseflies are an unlikely reservoir or vector for *Helicobacter pylori*." J Clin Microbiol 36(9): 2786-8.

Otrakji CL, Albores-Saavedra J and Martinez AJ (1990). "Gastric malignant lymphoma with superimposed amebiasis." Am J Gastroenterol 85(1): 72-5.

Palozza P and Krinsky NI (1992). "Astaxanthin and canthaxanthin are potent antioxidants in a membrane model." Arch Biochem Biophys 297(2): 291-5.

Parsonnet J (1993). "*Helicobacter pylori* and gastric cancer." Gastroenterol Clin North Am 22(1): 89-104.

Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N and Sibley RK (1991). "*Helicobacter pylori* infection and the risk of gastric carcinoma." N Engl J Med 325(16): 1127-31.

Peek RM, Jr., Wirth HP, Moss SF, Yang M, Abdalla AM, Tham KT, Zhang T, Tang LH, Modlin IM and Blaser MJ (2000). "*Helicobacter pylori* alters gastric epithelial cell cycle events and gastrin secretion in Mongolian gerbils." Gastroenterology 118(1): 48-59.

Peng H, Ranaldi R, Diss TC, Isaacson PG, Bearzi I and Pan L (1998). "High frequency of CagA+ *Helicobacter pylori* infection in high-grade gastric MALT B-cell lymphomas." J Pathol 185(4): 409-12.

Perkins SE, Fox JG, Marini RP, Shen Z, Dangler CA and Ge Z (1998). "Experimental infection in cats with a cagA+ human isolate of *Helicobacter pylori*." Helicobacter 3(4): 225-35.

Peterson RA, Danon SJ and Eaton KA (2001). "Comparison of gastritis and gastric epithelial proliferation in *Helicobacter heilmannii*-infected nude and BALB/c mice." Vet Pathol 38(2): 173-83.

Pettersson A and Lignell Å (1999). "Astaxanthin deficiency in eggs and fry of baltic salmon *(salmo salar*) with the M74 syndrome." Ambio 28: 43-46.

Phull PS, Green CJ and Jacyna MR (1995). "A radical view of the stomach: the role of oxygen-derived free radicals and anti-oxidants in gastroduodenal disease." Eur J Gastroenterol Hepatol 7(3): 265-74.

Quina MG (1998). "*Helicobacter pylori* infection and dyspepsia." Ital J Gastroenterol Hepatol 30 Suppl 3: S286-8.

Raderer M, Pfeffel F, Pohl G, Mannhalter C, Valencak J and Chott A (2000). "Regression of colonic low grade B cell lymphoma of the mucosa associated lymphoid tissue type after eradication of *Helicobacter pylori*." Gut 46(1): 133-5.

Rebouche CJ (1991). "Ascorbic acid and carnitine biosynthesis." Am J Clin Nutr 54(6 Suppl): 1147S-1152S.

Reed PI (1999). "Vitamin C, *Helicobacter pylori* infection and gastric carcinogenesis." Int J Vitam Nutr Res 69(3): 220-7.

Ross JS, Bui HX, del Rosario A, Sonbati H, George M and Lee CY (1992). "*Helicobacter pylori*. Its role in the pathogenesis of peptic ulcer disease in a new animal model." Am J Pathol 141(3): 721-7.

Rossi G, Rossi M, Vitali CG, Fortuna D, Burroni D, Pancotto L, Capecchi S, Sozzi S, Renzoni G, Braca G, Del Giudice G, Rappuoli R, Ghiara P and Taccini E (1999). "A conventional beagle dog model for acute and chronic infection with *Helicobacter pylori*." Infect Immun 67(6): 3112-20.

Ruiz-Bustos E, Sierra-Beltran A, Romero MJ, Rodriguez-Jaramillo C and Ascencio F (2000). "Protection of BALB/c mice against experimental *Helicobacter pylori* infection by oral immunisation with *H. pylori* heparan sulphate-binding proteins coupled to cholera toxin beta-subunit." J Med Microbiol 49(6): 535-41.

Sakagami T, Dixon M, O'Rourke J, Howlett R, Alderuccio F, Vella J, Shimoyama T and Lee A (1996). "Atrophic gastric changes in both *Helicobacter felis* and *Helicobacter pylori* infected mice are host dependent and separate from antral gastritis." Gut 39(5): 639-48.

Sakaki N, Arakawa T, Kozawa H, Yamada Y, Kato H, Kamisawa T and Momma K (1998). "Preliminary study on a novel quadruple eradication therapy with a mucoprotective drug, sofalcone, for *Helicobacter pylori* infection." J Clin Gastroenterol 27(Suppl 1): S187-91.

Sawai N, Kita M, Kodama T, Tanahashi T, Yamaoka Y, Tagawa Y, Iwakura Y and Imanishi J (1999). "Role of gamma interferon in *Helicobacter pylori*-induced gastric inflammatory responses in a mouse model." Infect Immun 67(1): 279-85.

Schmausser B, Mueller SO, Eck M, Moller M, Muller-Hermelink H and Stopper H (2000). "*Helicobacter pylori* induces DNA damage in vitro." Cancer Lett 152(2): 145-9.

Scott DR, Marcus EA, Weeks DL, Lee A, Melchers K and Sachs G (2000). "Expression of the *Helicobacter pylori* ureI gene is required for acidic pH activation of cytoplasmic urease." Infect Immun 68(2): 470-7.

Shahidi F, Metusalach and Brown JA (1998). "Carotenoid pigments in seafoods and aquaculture." Crit Rev Food Sci Nutr 38(1): 1-67.

Shibata K, Ito Y, Hongo A, Yasoshima A, Endo T and Ohashi M (1995). "Bacterial activity of a new antiulcer agent, ecabet sodium, against *Helicobacter pylori* under acidic conditions." Antimicrob Agents Chemother 39(6): 1295-9.

Shimizu N, Kaminishi M, Tatematsu M, Tsuji E, Yoshikawa A, Yamaguchi H, Aoki F and Oohara T (1998). "*Helicobacter pylori* promotes development of pepsinogen-altered pyloric glands, a preneoplastic lesion of glandular stomach of BALB/c mice pretreated with N-methyl-N-nitrosourea." Cancer Lett 123(1): 63-9.

Shinchi K, Ishii H, Imanishi K and Kono S (1997). "Relationship of cigarette smoking, alcohol use, and dietary habits with *Helicobacter pylori* infection in Japanese men." Scand J Gastroenterol 32(7): 651-5.

Shomer NH, Dangler CA, Whary MT and Fox JG (1998). "Experimental *Helicobacter pylori* infection induces antral gastritis and gastric mucosa-associated lymphoid tissue in guinea pigs." Infect Immun 66(6): 2614-8.

Siebenlist U, Franzoso G and Brown K (1994). "Structure, regulation and function of NF-kappa B." Annu Rev Cell Biol 10: 405-55.

Simpson KW, Strauss-Ayali D, Straubinger RK, Scanziani E, McDonough PL, Straubinger AF, Chang YF, Esteves MI, Fox JG, Domeneghini C, Arebi N and Calam J (2001). "*Helicobacter pylori* Infection in the Cat: Evaluation of Gastric Colonization, Inflammation and Function." Helicobacter 6(1): 1-14.

Sisto F, Brenciaglia MI, Scaltrito MM and Dubini F (2000). "*Helicobacter pylori*: ureA, cagA and vacA expression during conversion to the coccoid form." Int J Antimicrob Agents 15(4): 277-82.

Slomiany BL, Piotrowski J, Majka J and Slomiany A (1995). "Sucralfate affects the susceptibility of *Helicobacter pylori* to antimicrobial agents." Scand J Gastroenterol Suppl 210: 82-4.

Smythies LE, Waites KB, Lindsey JR, Harris PR, Ghiara P and Smith PD (2000). "*Helicobacter pylori*-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-gamma, gene-deficient mice." J Immunol 165(2): 1022-9.

Solnick JV, Canfield DR, Hansen LM and Torabian SZ (2000). "Immunization with recombinant *Helicobacter pylori* urease in specific- pathogen-free rhesus monkeys (Macaca mulatta)." Infect Immun 68(5): 2560-5.

Solnick JV, O'Rourke J, Lee A, Paster BJ, Dewhirst FE and Tompkins LS (1993). "An uncultured gastric spiral organism is a newly identified *Helicobacter* in humans." J Infect Dis 168(2): 379-85.

Soltez V, Schalén C and Mårdh PA (1988). New selective medium for *Campylobacter pylori*. Proceedings of the Fourth International Workshop on *Campylobacter* infections, Gothenburg, University of Gothenburg.

Sommer F, Faller G, Konturek P, Kirchner T, Hahn EG, Zeus J, Rollinghoff M and Lohoff M (1998). "Antrum- and corpus mucosa-infiltrating CD4(+) lymphocytes in *Helicobacter pylori* gastritis display a Th1 phenotype." Infect Immun 66(11): 5543-6.

Stahelin HB, Gey KF, Eichholzer M and Ludin E (1991). "Beta-carotene and cancer prevention: the Basel Study." Am J Clin Nutr 53(1 Suppl): 265S-269S.

Stanghellini V, Tosetti C, Barbara G, De Giorgio R, Salvioli B and Corinaldesi R (2000). "Review article: the continuing dilemma of dyspepsia." Aliment Pharmacol Ther 14 Suppl 3: 23-30.

Strauss-Ayali D, Scanziani E, Deng D and Simpson KW (2001). "*Helicobacter* spp. infection in cats: evaluation of the humoral immune response and prevalence of gastric *Helicobacter* spp." Vet Microbiol 79(3): 253-65.

Sturegard E, Sjunnesson H, Ho B, Willen R, Aleljung P, Ng HC and Wadstrom T (1998). "Severe gastritis in guinea-pigs infected with *Helicobacter pylori*." J Med Microbiol 47(12): 1123-9.

Sturegard E, Sjunnesson H, Nilsson H, Andersson R, Areskoug C and Wadstrom T (2001). "Infection with cagA- and vacA-positive and -negative strains of *Helicobacter pylori* in a mouse model." FEMS Immunol Med Microbiol 30(2): 115-20.

Sugiyama A, Maruta F, Ikeno T, Ishida K, Kawasaki S, Katsuyama T, Shimizu N and Tatematsu M (1998). "*Helicobacter pylori* infection enhances N-methyl-N-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil." Cancer Res 58(*10*): 2067-9.

Sutton P, Wilson J, Kosaka T, Wolowczuk I and Lee A (2000). "Therapeutic immunization against *Helicobacter pylori* infection in the absence of antibodies." Immunol Cell Biol 78(1): 28-30.

Suzuki H, Mori M, Seto K, Kai A, Kawaguchi C, Suzuki M, Suematsu M, Yoneta T, Miura S and Ishii H (1999). "*Helicobacter pylori*-associated gastric pro- and antioxidant formation in Mongolian gerbils." Free Radic Biol Med 26(5-6): 679-84.

Syder AJ, Guruge JL, Li Q, Hu Y, Oleksiewicz CM, Lorenz RG, Karam SM, Falk PG and Gordon JI (1999). "*Helicobacter pylori* attaches to NeuAc alpha 2,3Gal beta 1,4 glycoconjugates produced in the stomach of transgenic mice lacking parietal cells." Mol Cell 3(3): 263-74.

Tahara E (1998). "Molecular mechanism of human stomach carcinogenesis implicated in *Helicobacter pylori* infection." Exp Toxicol Pathol 50(4-6): 375-8.

Talley NJ, Zinsmeister AR, Weaver A, DiMagno EP, Carpenter HA, Perez-Perez GI and Blaser MJ (1991). "Gastric adenocarcinoma and *Helicobacter pylori* infection." J Natl Cancer Inst 83(23): 1734-9.

Tanaka T, Makita H, Ohnishi M, Mori H, Satoh K and Hara A (1995). "Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin." Cancer Res 55(18): 4059-64.

Tatematsu M, Yamamoto M, Shimizu N, Yoshikawa A, Fukami H, Kaminishi M, Oohara T, Sugiyama A and Ikeno T (1998). "Induction of glandular stomach cancers in *Helicobacter pylori*-sensitive Mongolian gerbils treated with N-methyl-N-nitrosourea and N-methyl-N'- nitro-N-nitrosoguanidine in drinking water." Jpn J Cancer Res 89(2): 97-104.

Taylor DW, Levander OA, Krishna VR, Evans CB, Morris VC and Barta JR (1997). "Vitamin E-deficient diets enriched with fish oil suppress lethal Plasmodium yoelii infections in athymic and scid/bg mice." Infect Immun 65(1): 197-202.

Testerman T, McGee D and Mobley HL (2001). Adherence and colonization. *Helicobacter pylori*: Physiology and Genetics. Mobley HLT, Mendz GL and Hazell SL. *Washington, D.C., ASM press*: 381-417.

Thomas JE, Gibson GR, Darboe MK, Dale A and Weaver LT (1992). "Isolation of *Helicobacter pylori* from human faeces [see comments]." Lancet 340(8829): 1194-5.

Thompson L, Cockayne A and Spiller RC (1994). "Inhibitory effect of polyunsaturated fatty acids on the growth of *Helicobacter pylori*: a possible explanation of the effect of diet on peptic ulceration." Gut 35(11): 1557-61.

Todd Lorenz R and Cysewski GR (2000). "Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin." Trends in Biotechnology 18(4): 160-7.

Todoroki I, Joh T, Watanabe K, Miyashita M, Seno K, Nomura T, Ohara H, Yokoyama Y, Tochikubo K and Itoh M (2000). "Suppressive effects of DNA vaccines encoding heat shock protein on *Helicobacter pylori*-induced gastritis in mice." Biochem Biophys Res Commun 277(1): 159-63.

Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Venter JC and et al. (1997). "The complete genome sequence of the gastric pathogen *Helicobacter pylori*." Nature 388(6642): 539-47.

Tompkins DS, Wyatt JI, Rathbone BJ and West AP (1988). "The characterization and pathological significance of gastric *Campylobacter*-like organisms in the ferret: a model for chronic gastritis?" Epidemiol Infect 101(2): 269-78.

Tsugane S, Tei Y, Takahashi T, Watanabe S and Sugano K (1994). "Salty food intake and risk of *Helicobacter pylori* infection." Jpn J Cancer Res 85(5): 474-8.

van Den Brink GR, ten Kate FJ, Ponsioen CY, Rive MM, Tytgat GN, van Deventer SJ and Peppelenbosch MP (2000). "Expression and activation of NF-kappa B in the antrum of the human stomach." J Immunol 164(6): 3353-9.

van Doorn NE, Namavar F, Sparrius M, Stoof J, van Rees EP, van Doorn LJ and Vandenbroucke-Grauls CM (1999). "*Helicobacter pylori*-associated gastritis in mice is host and strain specific." Infect Immun 67(6): 3040-6.

Van Oijen AH, Verbeek AL, Jansen JB and De Boer WA (2000). "Review article: treatment of *Helicobacter pylori* infection with ranitidine bismuth citrate- or proton pump inhibitor-based triple therapies." Aliment Pharmacol Ther 14(8): 991-9.

Vandamme P, Glupczynski Y, Lage AP, Lammens C, Quint WG and Goossens H (1995). "Evaluation of random and repetitive motif primed polymerase chain reaction typing of *Helicobater pylori*." Sys Appl Microbiol 18: 357-362.

Varis K, Taylor PR, Sipponen P, Samloff IM, Heinonen OP, Albanes D, Harkonen M, Huttunen JK, Laxen F and Virtamo J (1998). "Gastric cancer and premalignant lesions in atrophic gastritis: a controlled trial on the effect of supplementation with alpha-tocopherol and beta-carotene. The Helsinki Gastritis Study Group." Scand J Gastroenterol 33(3): 294-300.

Velioglu YS, Mazza G, Gao L and Oomah BD (1998). "Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products." J Agric Food Chem 46: 4113-7.

Wada T, Aiba Y, Shimizu K, Takagi A, Miwa T and Koga Y (1999). "The therapeutic effect of bovine lactoferrin in the host infected with *Helicobacter pylori*." Scand J Gastroenterol 34(3): 238-43.

Wadström T, Hirmo S and Borén T (1996). "Biochemical aspects of *Helicobacter pylori* colonization of the human gastric mucosa." Aliment Pharmacol Ther 10 Suppl 1: 17-27.

Wadström T and Aleljung P (1997). Oral presentation for the prophylactic and therapeutic treatment of *Helicobacter sp.* infection. Pat appl. WO 9837874.

Walker MM and Crabtree JE (1998). "*Helicobacter pylori* infection and the pathogenesis of duodenal ulceration." Ann N Y Acad Sci 859: 96-111.

Wang D, Kreutzer DA and Essigmann JM (1998). "Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions." Mutat Res 400(1-2): 99-115.

Wang J, Tauchi Y, Deguchi Y, Morimoto K, Tabata Y and Ikada Y (2000). "Positively charged gelatin microspheres as gastric mucoadhesive drug delivery system for eradication of *H. pylori*." Drug Deliv 7(4): 237-43.

Wang TC, Dangler CA, Chen D, Goldenring JR, Koh T, Raychowdhury R, Coffey RJ, Ito S, Varro A, Dockray GJ and Fox JG (2000). "Synergistic interaction between hypergastrinemia and *Helicobacter* infection in a mouse model of gastric cancer." Gastroenterology 118(1): 36-47.

Wang X, Sturegard E, Rupar R, Nilsson HO, Aleljung PA, Carlén B, Willén R and Wadström T (1997). "Infection of BALB/c A mice by spiral and coccoid forms of *Helicobacter pylori*." J Med Microbiol 46(8): 657-63. **[Paper I]**

Wang X, Aleljung P, Willén R and Wadström T (1998a). "Effects of antioxidants against *H. pylori* infection in BALB/cA mice." Gut (Suppl 2) 43: A32.

Wang X, Willén R and Wadström T (1998b). "Gastric Dysplasia due to *H. pylori i*nfection and carcinogenic substances in mice." Gut (suppl) 43(2): A69(08/238).

Wang X, Sjunnesson H, Sturegard E, Wadström T, Willén R and Aleljung P (1998). "Dietary factors influence the recovery rates of *Helicobacter pylori* in a BALB/cA mouse model." Zentralbl Bakteriol 288(2): 195-205. **[Paper II]**

Wang X, Willén R, Wadström T and Aleljung P (1998). "RAPD-PCR, histopathological and serological analysis of four mouse strains infected with multiple strains of *Helicobacter pylori*." Microbial Ecology in Health and Disease 10: 148-154. **[Paper III]**

Wang X, Willén R, Andersson C and Wadström T (2000). "Development of high-grade lymphoma in *Helicobacter pylori*-infected C57BL/6 mice." Apmis 108(7-8): 503-8. **[Paper IV]**

Wang X, Willén R and Wadstrom T (2000). "Astaxanthin-rich algal meal and vitamin C inhibit *Helicobacter pylori* infection in BALB/cA mice." Antimicrob Agents Chemother 44(9): 2452-7. **[Paper V]**

Wang X, Sjunnesson H, Sturegard E, Wadström T, Willén R and Aleljung P (2000). "Dietary factors influence the recovery rates of *Helicobacter pylori* in a BALB/cA mouse model." B&K Science Now 9(1): 1-6.

Wang X, Hirmo S, Willén R and Wadström T (2001). "Inhibition of *Helicobacter pylori* infection by bovine milk glycoconjugates in a BALB/cA mouse model." J Med Microbiol 50(5): 430-5.

Waring AJ, Drake IM, Schorah CJ, White KL, Lynch DA, Axon AT and Dixon MF (1996). "Ascorbic acid and total vitamin C concentrations in plasma, gastric juice, and gastrointestinal mucosa: effects of gastritis and oral supplementation." Gut 38(2): 171-6.

Warren JR and Marshall BJ (1983). "Unidentified curved bacilli on gastric epithelium in active chronic gastritis." Lancet 1(8336): 1273-5.

Watanabe T, Tada M, Nagai H, Sasaki S and Nakao M (1998). "*Helicobacter pylori* infection induces gastric cancer in mongolian gerbils." Gastroenterology 115(3): 642-8.

Weeks DL, Eskandari S, Scott DR and Sachs G (2000). "A H^+-gated urea channel: the link between *Helicobacter pylori* urease and gastric colonization." Science 287(5452): 482-5.

Wei H (1992). "Activation of oncogenes and/or inactivation of anti-oncogenes by reactive oxygen species." Med Hypotheses 39(3): 267-70.

Willen R, Carlen B, Wang X, Papadogiannakis N, Odselius R and Wadstrom T (2000). "Morphologic conversion of *Helicobacter pylori* from spiral to coccoid form. Scanning (SEM) and transmission electron microscopy (TEM) suggest viability [In Process Citation]." Ups J Med Sci 105(1): 31-40.

Wirth HP, Beins MH, Yang M, Tham KT and Blaser MJ (1998). "Experimental infection of Mongolian gerbils with wild-type and mutant *Helicobacter pylori* strains." Infect Immun 66(10): 4856-66.

Woodward M, Morrison C and McColl K (2000). "An investigation into factors associated with *Helicobacter pylori* infection." J Clin Epidemiol 53(2): 175-81.

Wotherspoon AC, Doglioni C, Diss TC, Pan L, Moschini A, de Boni M and Isaacson PG (1993). "Regression of primary low-grade B-cell gastric lymphoma of mucosa- associated lymphoid tissue type after eradication of *Helicobacter pylori*." Lancet 342(8871): 575-7.

Wyatt JI, Rathbone BJ, Dixon MF and Heatley RV (1987). "*Campylobacter pyloridis* and acid induced gastric metaplasia in the pathogenesis of duodenitis." J Clin Pathol 40(8): 841-8.

Xia HH and Talley NJ (1999). "*Helicobacter pylori* eradication in patients with non-ulcer dyspepsia." Drugs 58(5): 785-92.

Xia HH and Talley NJ (2001). "Apoptosis in gastric epithelium induced by *Helicobacter pylori* infection: implications in gastric carcinogenesis." Am J Gastroenterol 96(1): 16-26.

Yaquian E (2001). A study on the effects of antioxidants on *Helicobacter pylori*. Department of Applied Nutrition and Department of Medical Microbiology, Dermatology and Infection. Lund, Lund University.

Yee YK and Koo MW (2000). "Anti-*Helicobacter pylori* activity of Chinese tea: in vitro study." Aliment Pharmacol Ther 14(5): 635-8.

Yoshimura T and Johnson DG (1993). "cDNA cloning and expression of guinea pig neutrophil attractant protein- 1 (NAP-1). NAP-1 is highly conserved in guinea pig." J Immunol 151(11): 6225-36.

You WC, Zhang L, Gail MH, Chang YS, Liu WD, Ma JL, Li JY, Jin ML, Hu YR, Yang CS, Blaser MJ, Correa P, Blot WJ, Fraumeni JF, Jr. and Xu GW (2000). "Gastric dysplasia and gastric cancer: *Helicobacter pylori*, serum vitamin C, and other risk factors." J Natl Cancer Inst 92(19): 1607-12.

Young IS and Woodside JV (2001). "Antioxidants in health and disease." J Clin Pathol 54(3): 176-86.

Yu J, Russell RM, Salomon RN, Murphy JC, Palley LS and Fox JG (1995). "Effect of *Helicobacter mustelae* infection on ferret gastric epithelial cell proliferation." Carcinogenesis 16(8): 1927-31.

Zeng Z, Hu P and Chen M (1998). "[Development of mouse and rat model of *Helicobacter pylori* infection]." Zhonghua Yi Xue Za Zhi 78(7): 494-7.

Zhang HM, Wakisaka N, Maeda O and Yamamoto T (1997). "Vitamin C inhibits the growth of a bacterial risk factor for gastric carcinoma: *Helicobacter pylori*." Cancer 80(10): 1897-903.

Zhang Q, Dawodu JB, Etolhi G, Husain A, Gemmell CG and Russell RI (1997). "Relationship between the mucosal production of reactive oxygen radicals and density of *Helicobacter pylori* in patients with duodenal ulcer." Eur J Gastroenterol Hepatol 9(3): 261-5.

Zhang ZW, Patchett SE, Perrett D, Domizio P and Farthing MJ (2000). "Gastric alpha-tocopherol and beta-carotene concentrations in association with *Helicobacter pylori* infection." Eur J Gastroenterol Hepatol 12(5): 497-503.

Zhang ZW, Patchett SE, Perrett D, Katelaris PH, Domizio P and Farthing MJ (1998). "The relation between gastric vitamin C concentrations, mucosal histology, and CagA seropositivity in the human stomach." Gut 43(3): 322-6.

Zheng PY, Hua J, Ng HC and Ho B (1999). "Unchanged characteristics of *Helicobacter pylori* during its morphological conversion." Microbios 98(389): 51-64.

J. Med. Microbiol. — Vol. 46 (1997), 657–663

MODELS OF INFECTION

I

Infection of BALB/c A mice by spiral and coccoid forms of *Helicobacter pylori*

X. WANG, E. STUREGARD, R. RUPAR, H-O. NILSSON, P. A. ALELJUNG, BIRGITTA CARLÉN*, R. WILLÉN* and T. WADSTROM

*Departments of Medical Microbiology and *Pathology, University of Lund, S-223 62 Lund, Sweden*

Helicobacter pylori exists in two different morphological forms, spiral and coccoid. This study demonstrated that both forms can infect BALB/c A mice. The animals were inoculated orally three times at 2-day intervals with 10^8 cfu of both spiral and coccoid forms of strain CCUG **17874** (NCTC 11637), strain 25 and strain 553/93. Infection was followed over a 30-week period by histological scoring of the grade of inflammation in gastric biopsies. At each time point sera were collected for analysis in ELISA and immunoblot analysis. Both spiral and coccoid forms of all ***H. pylori*** strains gave significantly higher inflammation scores than a control group of animals 1 week after inoculation. The histological evidence persisted throughout the entire 30 weeks. The inflammation was most severe in the pylorus and duodenum. Infection with strain 553/93 displayed the most severe gastritis. The spiral form of strain CCUG **17874** gave an immune response after only **4** weeks, whereas its coccoid form as well as strains 25 and 553/93 (spiral and coccoid forms) gave a significant increase in antibody response in ELISA and immunoblot after **16** weeks. It is concluded that both spiral and coccoid forms of ***H. pylori*** can cause acute gastritis in BALB/c A mice.

Introduction

Helicobacter pylori is a human pathogen associated with type B gastritis, peptic ulcer disease and gastric cancer [1–3]. In recent years an increasing number of animal models has been used to study the pathogenesis of *H.* pylori infection. Oral challenges with *H.* pylori resulted in infection in monkeys, gnotobiotic pigs and nude and euthymic mice [4–6]. Marchetti et *al.* [7] recently reported that vacuolating toxin (VAC)-producing strains of *H.* pylori can colonise the murine stomach and induce histopathological changes similar to type B gastritis in man.

H. pylori exists in two different morphological forms, spiral and coccoid. Coccoid forms of *H.* pylori have been described as 'viable but non-culturable' (VBNC). These cells may be viable and can revert to culturable forms in mice, but are no longer culturable on conventional media [8, 9]. The role of the coccoid form in the pathogenesis of *H.* pylori-associated gastritis has been disputed [10, 11]. A recent study demonstrated chronic gastrointestinal colonisation of mice with *H.* pylori strains expressing high heparin-binding activity [12]. The present study describes the further development of this model in which BALB/c A mice were infected and the histopathological changes and systemic responses were followed at 1, 2, 4, 8, 16, 24 and 30 weeks.

Received 24 Sept. 1996: revised version acceoted 10 Jan. 1997.
Corresponding author: Professor T. Wadstrom.

Materials and methods

Bacterial strains and culture

H. pylori strain 17874 was obtained from the Culture Collection, University of Gothenburg (CCUG), Sweden (identical with strain NCTC 11637 from the National Collection of Type Cultures, 61 Colindale Avenue, London). *H. pylori* strain 25 [13] and strain 553/93 were freshly isolated from human gastric biopsies. The strains were grown on GAB-CAMP agar supplemented with horse serum 10% [14, 15] and were incubated for 48 h at 37°C in micro-aerophilic conditions to obtain a maximal yield of spiral-shaped *H.* pylori [12]. To obtain viable but non-culturable *H.* pylori cells, growth from 3–5-day-old agar cultures was harvested and resuspended in 20 ml of Ham's F12 medium supplemented with calf serum (Flow Laboratories, Irvine) 10% and kept in a micro-aerobic environment for 3

days at 37°C and kept at 4°C. If no growth was observed after incubation for 5 days on GAB-CAMP agar at 37°C, harvested cells were defined as viable but non-culturable [12]. All bacteria were checked for the presence of spiral forms in the coccoid suspension and *vice versa:* the spiral form was not found in the coccoid suspension whereas only a few coccoid forms were observed among the spiral suspension (< 0.001%).

Animals

BALB/c **A** mice (6–8 weeks old) were used in this study [12]. Mice were housed with a 12-h light-dark schedule, fed a commercial rodent diet (B&K Universal AB, Sweden) and provided with water *ad libitum*.

Experimental design

Mice were inoculated orally through a feeding tube (OD, 0.1 mm) three times at 2-day intervals with 0.1 ml of either bacterial suspension (10^9 cfu/ml) or PBS. One hundred and fifty mice were divided into seven groups. Three groups of mice were inoculated with suspensions of strain CCUG 17874, strain 25 and strain 553/93, respectively. This experimental design was repeated with suspensions of the coccoid form of *H. pylori;* the control group received PBS. Three or four mice from each group were killed at 1, 2, 4, **8,** 16, 24 and 30 weeks after inoculation. Mice were anaesthetised with ether and killed before collection of their stomachs.

The stomach was opened through the longer curvature with sterile surgical instruments. One half of the stomach and duodenum, covering all subtypes of mucosa, was sent for histopathological examination. The rest of the stomach was used for culture and PCR. Blood was drawn for measurement of the immune response.

Culture

Gastric mucosa samples were homogenised with PBS; then 100 μl of homogenate were placed on GAB-camp agar and incubated for 7 days in micro-aerobic conditions at 37°C. The presence of *H. pylori* on the culture plates was confirmed by the urease, catalase and oxidase tests, Gram's staining and PCR [16].

Histopathological examination

Each stomach was fixed in buffered formalin 10% (effective osmotic pressure 300 mosm/L) and embedded in paraffin. Sections (4 μm) were prepared and stained with haematoxylin and eosin, following standard procedures. Five areas of stomach were examined (Fig. 1); fundic mucosa with its stratified keratinised, squamous epithelium; cardia in the transitive zone between fundus and corpus; body; antrum and canalis; and duodenum. The degree of inflammation was scored between 0 and *3* in the five different parts of the stomach and duodenum. The inflammatory infiltrate comprised mainly granular cells: 0, normal; 1, few inflammatory cells; 2, moderate inflammatory cells in several layers; *3,* high level of inflammation with foci containing > 50 inflammatory cells, often more than three cell layers deep.

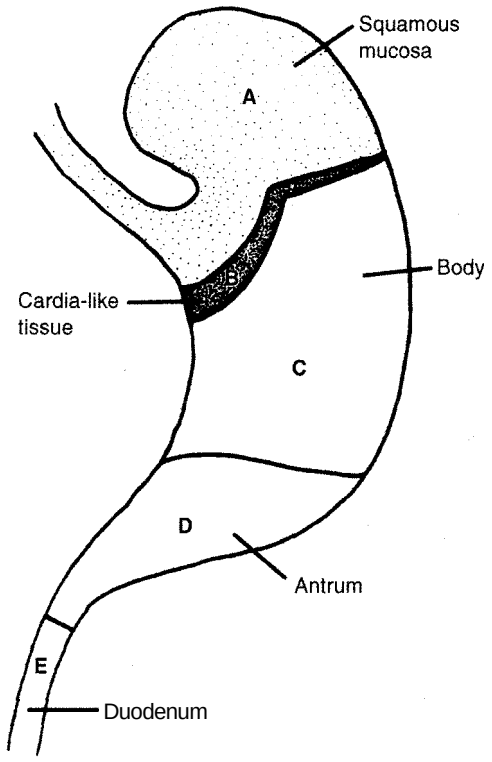


Fig. 1. Diagram of the mouse stomach. **A–E** are the five areas examined by histology.

Transmission electron microscopy

The biopsy specimens were fixed immediately in glutaraldehyde 2% in 0.1 M sodium cacodylate buffer (pH 7.2), post-fixed in osmium tetroxide 2% in S-collidine buffer (pH 7.2), dehydrated in ethanol and embedded in agar resin 100. A semi-thin section was cut and examined by light microscopy. A representative area was chosen and ultra-thin sections, c. 50 nm, were cut on an LKB Ultratome III and contrasted with uranyl acetate and lead citrate. The grids were examined in a Zeiss CM 10 electron microscope at 60 kV.

ELISA and immunoblot

Sera were examined for total antibodies (mainly IgG) to *H. pylori* by ELISA as described by Guruge [15]. Serum samples (100 μl diluted 1 in 200) were added to each well, which had been coated with *H. pylori* antigen (acidic glycine extract) [17]. After incubation for 90 min at 37°C, wells were washed and 100 μl of HRP-labelled goat anti-mouse immunoglobulins (Dakopatts, Copenhagen, Denmark) diluted 1 in 2000 in the washing buffer were added. The plates were

incubated for 60 min at 37°C, followed by addition of substrate and stopping solution, and absorbance values at 450 nm were measured in a spectrophotometer.

Sera diluted 1 in 50 in washing buffer (10 mM Tris base, 30 mM NaCl, 1 mM NaN_3, pH 10.2 and Tween 20 0.1%) were incubated with strips from SDS-PAGE and blotted against a glycine extract of *H. pylori* overnight on a shaker at 4°C [15]. Anti-mouse immunoglobulins labelled with HRP (diluted 1 in 600) were added for 3 h on a shaker at 4°C and developed for 30–40 min with 50 mM sodium acetate containing carbazole 0.02% and H_2O_2 0.3% at room temperature.

DNA extraction and PCR conditions

DNA was extracted from frozen homogenates of gastric biopsies from control and *H.* pylori-infected mice: 100 μl of homogenate were centrifuged at 12000 *g* for 5 min and resuspended in 380 μl of TNE buffer with Triton X-100 1% and lysozyme 0.5 mg/ml. The samples were incubated at 37°C for 30 min, proteinase K was added to 1 mg/ml and incubated overnight at 37°C. DNA was isolated by extracting twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform. DNA was precipitated with 0.3 M sodium acetate and 2 volumes of absolute ethanol and centrifuged as described above, rinsed in alcohol 70% and dried in a speed vacuum [18]. The DNA pellet was dissolved in 50 μl of water plus Tween and stored at –20°C until the PCR was performed.

HPU primers, based on a urease **A** gene sequence of *H.* pylori, as originally described by Clayton *et al.* [19] were used. Primers were purchased from Scandinavian Gene Synthesis (Koping, Sweden). PCR conditions were according to Nilsson *et al.* [16]

Statistical analysis

The Mann-Whitney U test was used to compare the degree of inflammation and the changes in immune response. The level of significance selected was $p < 0.05$.

Results

In the infected groups, there was histological evidence of type B gastritis with infiltration of polymorphonuclear leucocytes (PMNL) or lymphocytes, or both, persisting for 30 weeks. In contrast, the uninfected control group and the group inoculated with formalin-killed *H. pylori* showed no evidence of gastritis. In several mice it was possible to demonstrate *H. pylori* in both spiral and coccoid form. Electron microscopy demonstrated well-preserved subcellular and membrane structures, giving the impression of viable bacteria. It was possible to demonstrate a clear attachment to the mucosal cell surface membrane, especially around the coccoid form, and a tendency for pedestal formation was noticed (Fig. 2).

Both the spiral and the coccoid forms of *H. pylori* strains CCUG 17874 and 25 gave a significantly higher inflammation score in the gastric biopsies of mice 1 week after inoculation. For *H. pylori* strain 553/93, both spiral and coccoid forms gave more severe inflammation than the other strains tested (Fig. 3).

Pyloric and duodenal inflammation was most severe in infected groups compared to the control group. Finally, strain 553/93 caused a significantly higher inflammation score than the other types tested (Fig. 4).

The colonisation of mouse stomach by *H. pylori* was investigated by culture and PCR. The colonies on the GAB-camp agar and also DNA extraction from mouse stomach both showed a H. pylori urease-specific band (Fig. 5).

H. pylori strain CCUG 17874 produces vacuolating cytotoxin (VacA) and expresses cytotoxin-associated gene (CagA) protein. Strain 25, which was isolated from man and used for several years in our laboratory is VacA-positive and CagA-positive; and strain 553/93, which was freshly isolated from man is both VacA- and CagA-negative (data not shown).

Spiral forms of strain CCUG 17874 gave a significant increase in antibody response in ELISA 4 weeks after inoculation ($p < 0.05$). Eight weeks after inoculation the immune response showed a much stronger response than at 4 weeks ($p < 0.05$). Strain 553/93 spiral forms gave a small increase 8 weeks after inoculation and at 16 weeks gave significantly higher titres ($p < 0.05$). Strain 25, both spiral and coccoid forms, gave an increase 16 weeks after inoculation ($p < 0.05$). Coccoid forms of strains 17874 and 553/93 gave the same changes as strain 25 (Fig. 6).

Immunoblot analysis showed an increase in the number of bands and intensity, especially of proteins in the 14–20 and 60–80 kDa region (Fig. 7). the immunoblot results of the different groups showed similar changes to the ELISA titres.

Discussion

H. pylori infections occur in human populations throughout the world. Once acquired, the infection becomes chronic and probably persists for life if untreated [20]. Multiple lines of evidence demonstrate the casual role of *H. pylori* in the chronic inflammatory process. Ingestion of *H. pylori* by human volunteers resulted in gastritis, and eradication of *H. pylori* infection resolved this gastritis [21]. Oral challenges

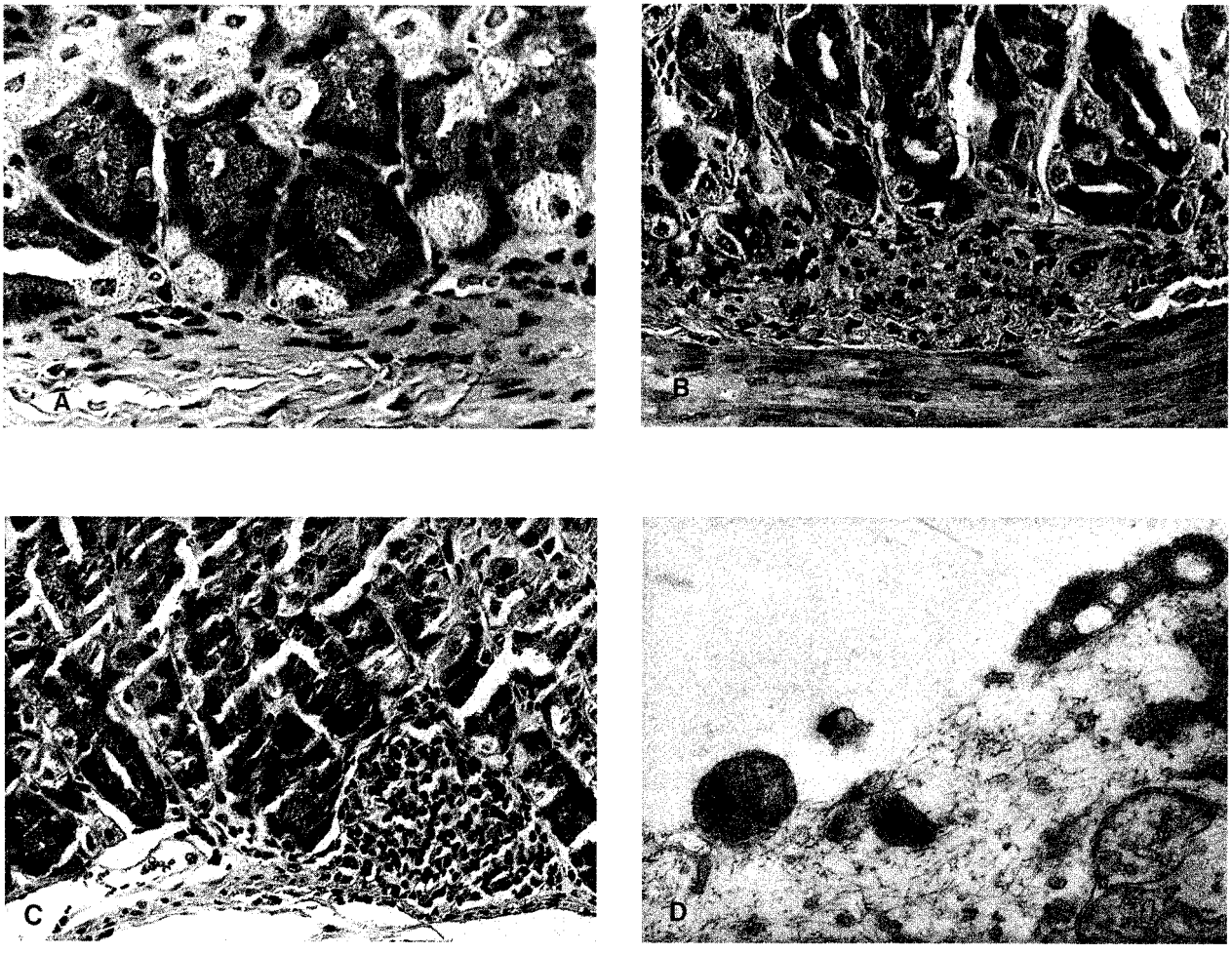


Fig. 2. Histopathological analysis of mouse stomachs. **(A)** No inflammation at base, from a control mouse (×280); **(B)** severe inflammation at glandular base of 16 weeks after inoculation with *H. pylori* strain 553/93 (spiral form) (×280); **(C)** severe inflammation at base with formation of lymphoid follicle 16 weeks after inoculation with *H. pylori* strain 553/93 (coccoid form) (×280). **(D)** Electron micrograph: mouse stomach infected with *H. pylori* spiral form (upper right) and coccoid form (lower left); note the tendency for pedestal formation, especially around the coccoid form (X24 000).

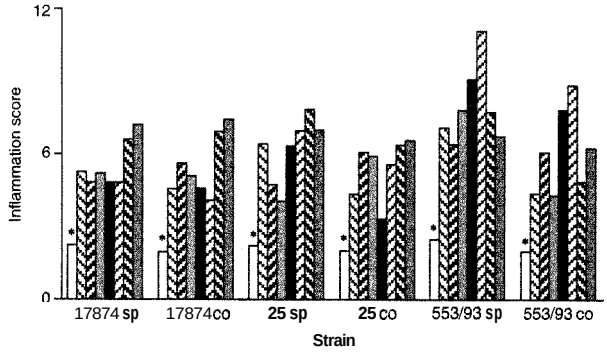


Fig. 3. Inflammation score in the stomach of BALB/c A mice infected by different *H. pylori* strains; □ control, ▨ 1 week, ▨ 2 weeks, ▨ 4 weeks, ■ **8** weeks, ▨ 16 weeks, ▨ 24 weeks, ▨ 30 weeks. *p < 0.05 ***versus*** all infected groups through the whole 30 weeks.

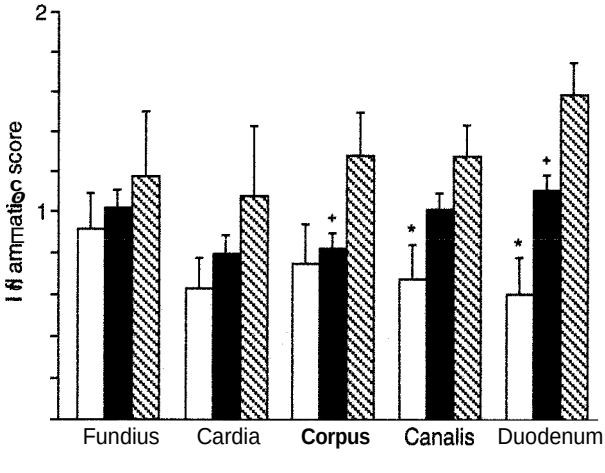


Fig. 4. Inflammation score in different regions of stomach; + and *p < 0.05 ***versus*** the group infected by strain 553/93 (fresh isolate strain from human). Inflammation scores **of** jejunum, ileum, colon, liver and lung were all zero; □ control, ■ all infected group, infected by human isolates.

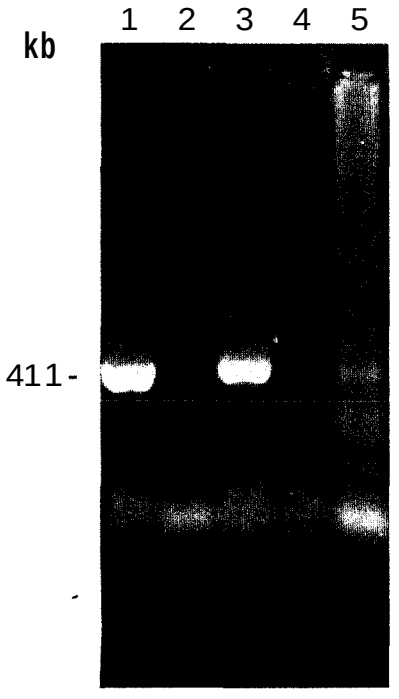


Fig. 5. PCR analysis. Lane **1,** positive control; **2,** negative control: **3,** colony on GAB-camp agar from infected mouse stomach; **4,** homogenate of stomach from control mice; **5,** homogenate of stomach from infected mice.

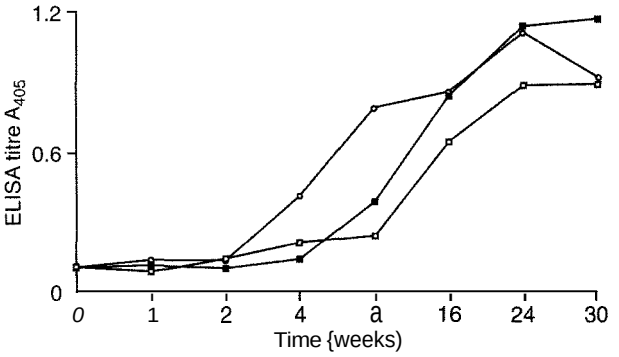


Fig. 6. Antibody response in BALB/c A mice to different *H. pylori* strains. Strain 17874 spiral form (○) gave a significant increase 4 weeks after inoculation and much higher at 8 weeks after inoculation. Strain 553/93 spiral form (■) gave a significantly higher titre **16** weeks after inoculation and strain 25 spiral form (∪) gave a significant change **16** weeks after inoculation. ** All coccoid forms of these three strains showed the same results as strain 25 spiral forms in ELISA titre.

with *H. pylori* also resulted in gastritis in monkeys, piglets and rodents [4, 5, 12].

H. *pylori* can exist in two forms, spiral and coccoid forms. Eaton *et al.* [22] reported that gnotobiotic piglets infected with spiral *H.* pylori developed lymphocytic gastritis and *H.* pylori-specific antibody but these changes were seen with coccoid forms followed for 14 days after challenge. Cellini's study [9] showed that coccoid *H.* pylori non-culturable in

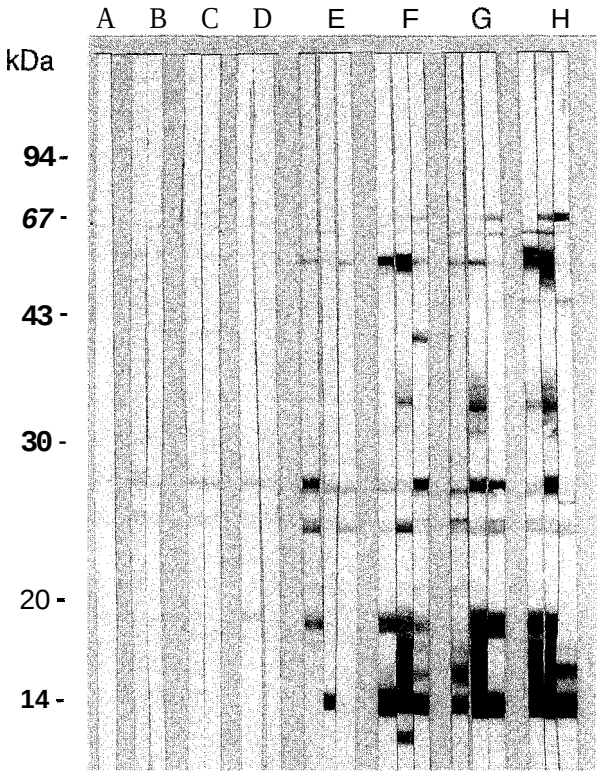


Fig. 7. Immunoblot of mice sera from the group infected by strain 553/93 spiral form. Lane **A,** control mouse; **B,** 1 week after inoculation; **C,** 2 weeks; **D,** 4 weeks; **E,** 8 weeks; **F**, 16 weeks; ***G*,** 24 weeks; **H,** 30 weeks after inoculation. Similar patterns can be seen in the other infected groups, but the group infected with strain 17874 spiral form already showed an increase at 4 weeks after inoculation.

vitro had reverted in mice 2 weeks after inoculation and all colonised mice showed a systemic antibody response to *H.* pylori. Coccoid forms of *H.* pylori can also exist in the human stomach and they were found more frequently and in larger numbers in cases of adenocarcinoma than in cases of benign ulcers [23]. In the present study, both spiral and coccoid forms of *H.* pylori gave a significant increase in inflammatory cells in stomach biopsy samples from BALB/c **A** mice 1 week after inoculation and the inflammation continued throughout the 30 weeks of the study (Fig. *3).* The inflammatory cells were mainly granular cells in this study. Inflammation was most severe in the pylorus and duodenum among infected animals compared to the control mice. Of the three strains of H. pylori used in this study, strain 553/93 caused a significantly higher inflammation score than the other two strains tested (Fig. 4). The distribution of *H.* pylori in the human stomach seems to have implications with respect to the disease process, and it has been suggested that the degree of gastritis can be used as an indicator of *H.* pylori density [24–26]. Danon *et al.* [27] observed that *H. felis* was mainly confined to the non-acid-producing regions, i.e., antrum and cardia, of the mouse stomach.

In spite of the advances in treatment and in the understanding of the epidemiology of type B gastritis, the mechanisms of *H. pylori* pathogenicity are still not well understood. Among many important virulence factors, the production of the vacuolating cytotoxin (VacA) and the expression of the cytotoxin-associated gene (CagA) protein were almost the first phenotypic characteristics described. However, these factors were not always present in all *H. pylori* strains. Thus, it is highly probable that they are associated with peptic ulcer disease, lymphoid proliferation and possibly gastric cancer. *H. pylori* strains producing the VacA toxin and CagA protein induced a more severe inflammation within the gastric mucosa in patients with peptic ulcer disease cases [7, 28]. The expression of CagA in the regulation of the inflammatory response to *H. pylori* infection is associated with increased secretion of interleukin-8 by gastric epithelial cells [29, 30]. Furthermore, CagA protein expression is also associated with an increased risk for development of gastric cancer [31]. *H. pylori* strain CCUG 17874 had commonly been recognised as a vacuolating cytotoxin (VacA)-producing and cytotoxin-associated gene (CagA) protein expressing strain [32]; strain 25 is a VacA-positive and CagA-positivestrain; and strain 553/93 which was freshly isolated from man **is** both VacA- and CagA-negative. Gastroduodenal inflammation in the mice can be caused by both cytotoxic and non-cytotoxic strains of *H. plori.*

Bacillary forms of strain 17874 gave a significant immune response 4 weeks after inoculation, while its coccoid forms gave a higher antibody response 16 weeks after inoculation. Strain 25 and 553/93 (both spiral and coccoid form) gave similar results to the coccoid form of strain 17874.

Finally, the study showed that both spiral and coccoid forms of *H. pylori* can induce gastritis in the gastric mucosa of BALB/c **A** mice and can produce an antibody response in the sera of infected mice. The finding that the coccoid form of *H. pylori* is active in the inflammatory process warrants further studies, which are now being done in our laboratory.

This study was supported by grants from the Swedish Medical Research Council (16X04723), Forssmans Fondation and Founds of Medical Faculty, University of Lund, Sweden.

References

1. Warren JR, Marshall B. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983; **1** 1273–1275.
2. Northfield TC, Mendall M, Goggin PC. *Helicobacter pylori:* infection, pathophysiology, epidemiology and management. Dordrecht, The Netherlands, Kluwer Academic Press 1994.
3. Wadstrom T. **An** update on *Helicobacter pylori. Curr Opin Gastroenterol* 1995; **11:** 69–75.
4. Dubois A, Fiala N, Heman-Ackah **NL** *et al.* Natural gastric infection with *Helicobacter pylori* in monkeys: a model for spiral bacteria infection in humans. *Gastroenterology* **106:** 1405–1417.
5. Engstrand L, Gustavsson **S**, Jorgensen A, Schwan A, Scheyenius A. Inoculation of barrier-born pigs with *Helicobacter pylori:* a useful animal model for gastritis type B. *Infect Immun* 1995; **58**: 1763–1768.
6. Karita M, Kouchiyama T, Okita K, Nakazawa T. New small animal model for human gastric *Helicobacter pylori* infection: success in both nude and euthymic mice. *Am J Gastroenterol* 1991; **86**: 1596–1603.
7. Marchetti M, Arico B, Burroni D, Figura N, Rappuoli R, Ghiara P. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science* 1995; **267:** 1655–1658.
8. Oliver JD, The viable but non-culturable state in the human pathogen *Vibro vulnificus. FEMS Microbiol Lett* 1995; **133:** 203–208.
9. Cellini L, Allocati N, Angelucci D *et al.* Coccoid *Helicobacter pylori* not culturable ***in*** *vitro* reverts in mice. *Microbiol Immunol* 1994; **38:** 843–850.
10. Graham DY, Lew GM, Malaty HM *et al.* Factors influencing the eradication of *Helicobacter pylori* with triple therapy. *Gastroenterology* 1992; **102:** 493–496.
11. Marshall BJ, Goodwin CS, Warren JR *et al.* Prospective double-blind trial of duodenal ulcer relapse after eradication of *Campylobacter pylori. Lancet* 1988; **2:** 1437–1442.
12. Aleljung **P**, Nilsson HO, Wang X *et al.* Gastrointestinal colonisation of BALB/c A mice by *Helicobacter pylori* monitored by heparin magnetic separation. *FEMS Immunol Med Microbiol* 1996; **13:** 303–309.
13. Lelwala-Guruge J, Ljungh Å, Wadstrom T. Haemagglutination patterns of *Helicobacter pylori.* Frequency of sialic acid-specific and non-sialic acid-specific haemagglutinins. *APMIS* 1992; **100:** 908–913.
14. Soltez V, Schalen C, Mårdh PA. New selective medium for *Campylobacter pylori.* In: Kaijser B, Falsen E (eds) Proceedings of the Fourth International Workshop on *Campylobacter* infections. University of Gothenburg, Gothenburg, Sweden. 1988: 433–436.
15. Lelwala-Guruge J, Schalén C, Nilsson I *et al.* Detection of antibiotics to *Helicobacter pylori* cell surface antigens. *Scand J Infect Dis* 1990; **22:** 457–465.
16. Nilsson HO, Aleljung P, Nilsson I, Tyszkiewicz T, Wadstrom T. Immunomagnetic bead enrichment and PCR for detection of *Helicobacter pylori* in human stools. *FEMS Immunol Med Microbiol* 1996; **27**: 73–79.
17. Lalwala-Guruge J, Nilsson I, Ljungh Å, Wadstrom T. Cell surface proteins of *Helicobacter pylori* as antigens in ELISA and a comparison with three commercial ELISA, *Scand J Infect Dis* 1992; **24:** 457–465.
18. Kong L, Smith JG, Bramhill D *et al.* **A** sensitive and specific PCR method to detect *Helicobacter felis* in a conventional mouse model. *Clin Diagn Lab Immunol* 1996; **3:** 73–78.
19. Clayton CL, Kleanthous H, Coates PJ, Morgan DD, Tabaqchali S. Sensitive detection of *Helicobacter pylori* by using polymerase chain reaction. *J Clin Microbiol* 1992; **30:** 192–200.
20. Blaser MJ. Hypotheses on the pathogenesis and natural history of *Helicobacter* pylori-induced inflammation. *Gastroenterology* 1992; **102**: 720–727.
21. Cover TL, Blaser MJ. *Helicobacter pylori:* a bacterial cause of gastritis, peptic ulcer disease, and gastric cancer. *ASM News* 1995; **61:** 21–26.
22. Eaton KA, Catrenich CE, Makin KM, Krakowka S. Virulence of coccoid and bacillary forms of *Helicobacter pylori* in gnotobiotic piglets. *J Infect Dis* 1995; **171** 459–462.
23. Chan WY, Hui PK, Leung KM, Chow J, Kwok F, Ng CS. Coccoid forms of *Helicobacter pylori* in the human stomach. *Am J Clin Pathol* 1994; **102:** 503–507.
24. Louw JA, Falck V, van Rensburg C, Zak J, **Adams** G, Marks IN. Distribution of *Helicobacter pylori* colonisation and associated gastric inflammatory changes: difference between patients with duodenal and gastric ulcers. *J Clin Pathol* 1993; **46:** 754–756.
25. McNulty CAM, Watson DM. Spiral bacteria of the gastric antrum. *Lancet* 1984; **1:** 1068–1069.
26. Satoh K, Kimura K, Yoshida Y, Kasano T, Kihira K, Taniguchi Y. A topographical relationship between *Helicobucter pylori* and gastritis: quantative assessment of *Helicobacter* ***pylori*** in the gastric mucosa. ***Am*** *J Gastroenterol* 1991; ***86*** 285–291.
27. Danon SJ, O'Rourke JL, Moss ND, Lee **A.** The importance of local acid production in the distribution **of** *Helicobacter*

felis in the mouse stomach. *Gastroenterology* 1995; **108:** 1386–1395.
28. Rautelin H, Blomberg B, Jarnerot G, Danielsson D. Nonopsonic activation of neutrophils and cytotoxin production by *Helicobacter pylori:* ulcerogenic markers. *Scand J Gastroenterol* 1994; **29:** 128–132.
29. Crabtree JE, Covacci **A,** Farmery SM *et al. Helicobacter pylori* induced interleukin-8 expression in gastric epithelia1 cells is associated with CagA positive phenotype. *J Clin Pathol* 1995; **48:** 41–45.
30. Yamaoka Y, Kita M, Kodama T, Sawai N, Imanishi J. *Helicobacter pylori* cagA gene and expression **of** cytokine messenger RNA in gastric mucosa. *Gastroenterology* 1996; **110:** 1744–1752.
31. Kuipers EJ, Péréz-Péréz **GI,** Meuwissen **SGM,** Blaser MJ. *Helicobacter pylori* and atrophic gastritis: importance of the cagA status. *J Natl Cancer Inst* 1995; **87:** 1777–1780.
32. Xiang Z, Censini S, Bayeli PF ***et al.*** Analysis of expression of CagA and VacA virulence factors in 43 strains **of** *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that cagA is not necessary for expression of the vacuolating cytotoxin. *Infect Immun* 1995; ***63:*** 94–98.

Zent.bl. Bakteriol. 288, 195–205 (1998)

Zentralblatt für
Bakteriologie

Dietary Factors Influence the Recovery Rates of *Helicobacter pylori* in a BALB/cA Mouse Model

Xin Wang[1], Håkan Sjunnesson[1], Erik Sturegård[1], Torkel Wadström[1]*, Roger Willén[2], and Par Aleljung[1]

[1] Department of Infectious Diseases and Medical Microbiology
[2] Department of Pathology Lund University, S-223 62 Lund, Sweden

Received November 28, 1997 . Revision received January 26, 1998 . Accepted March 12, 1998

Summary

The aim of this study was to assess the ability of different mouse diets to sustain an *H*. pylori infection in BALB/cA mice. Four commercially available mouse diets were compared. Experiment 1: Mice were fed the four diets for seven days before infection, infected three times at two-day intervals with 0.1 ml of 10^9 colony-forming units/ml *H*. pylori cells. *H*. pylori strains (n = 4) were cultured on GAB-Camp agar for 2 days, harvested and suspended in PBS. All animals were sacrificed at 2 and 4 weeks post inoculation. Experiment 2: Mice infected for 8 weeks were fed RM2, changed to the different diets for 10 days and sacrificed. Stomachs were collected, cultured on GAB-Camp agar to estimate *H*. pylori growth and stomach biopsies were analyzed by PCR. There were significant differences between diets in their ability to sustain growth of *H*. pylori. The range was from a few hundred colonies to no growth at all on the GAB-Camp agar. PCR signals showed good correlation with the culture results. All *H*. pylori-infected mice gave a significantly higher inflammation score compared to non-infected mice. The diet RM2, having the highest number of culturable H. pylori in the mouse stomach, also showed the highest inflammation. These results suggest that the dietary factors affect the amounts of *H*. pylori in an infection of BALB/cA mice.

* Corresponding author.

Introduction

Food is mainly associated with pleasure but there are significant negative side effects from its excessive consumption or maltreatment or intake of a nutritionally poor diet. There are other aspects of our diet that affect our immune functions, resistance to infections and cancer development (3, 12, 18, 20, 25, 32). Dietary factors have received considerable attention for the risk of developing gastric cancer in epidemiological studies and in animal models (5, 16) but also for the protective role diet plays in some infections (6, 8, 10). However, certain dietary components, polyunsaturated fatty acids, have an adverse effect on the laboratory animal (mouse)when infected with *Listeria monocytogenes* and *Salmonella typhimurium* (4, 9).

Helicobacter pylori causes chronic infection predisposing to type B gastritis, peptic ulcer disease, stomach atrophy and gastric cancer in man (7, 11). A working group of the WHO International Agency for Research on Cancer, concluded in 1994 that *H. pylori* is a Class I carcinogen in humans (15). Epidemiological investigations of various populations identify several dietary risk factors for gastric cancer, inappropriate food storage, salty foods, soybean products whereas high intake of fruits and vegetables or antioxidants (beta-carotene, C and E vitamins) seems to decrease the risks (14, 22, 29, 30). *Aldoori* et al. (1) found that dietary fibre and vitamin A may reduce the development of duodenal ulcer, but they could not exclude the possibility that other closely correlated dietary factors might be the true protective factors. Other dietary components like unsaturated fatty acids and garlic extract exhibited in-vitro activity against *H. pylori* (13, 28, 33).

The aim of this study was to compare how different commercial mouse diets affected the *H. pylori* infection in a BALB/cA mouse model and to develop a standardized diet in order to follow the development of chronic type B gastritis.

Materials and Methods

Diets. Four commercial mouse diets were investigated in this study. 1)Rat and mouse standard diet No. 1 *(RMI)*,2) Rat and mouse standard diet No. 2 *(RM2)*; 3) Rat and mouse autoclavable diet *(RMA)*autoclaved according to the manufacturers instructions; and 4)Rodent standard diet *(SDS)*[1–3 from B&K Universal, Sollentuna, Sweden, 4 from SDS, London, UK]. The differences of these diets are shown in Table 1 (according to the manufacturers' descriptions of the contents).

Animals. Conventional BALB/cA mice six to eight weeks old were used in this study (2). Mice were housed with a 12-hour light-dark schedule, fed different diets and given water *ad libitum*.

Bacterial Strains. H. pylori strains 553/93, 92/95, 134195, 92/96 (a cocktail of cagA and vacA positivelnegative strains) were grown in GAB-Camp agar supplemented with 10% horse serum (26) and incubated for 48 hours at 37°C under microaerobic conditions. The cells were harvested in PBS, centrifuged at 3000rpm for 10 minutes

Table 1. Some differences of the media investigated according to the manufacturers' description of contents. * RMA, RM1 and RM2 were obtained from B&K Universal Company whereas # SDS diet was obtained from SDS Company

Fixed formula/ Diets	Autoclavable diet (RMA)'	Standard No. 1 diet (RM1)[1]	Standard No. 2 diet (RM2)[1]	Standard diet (SDS)[2]
Wheat meal	+	+	+	+
Soya meal (%)	+ (14%)	+ (24%)	+ (20.8%)	+ (11%)
Barley meal	+	+	+	+
Wheatfeed	+	+	+	+
Fish meal	+	+	–	+
Full fat Soya	+	–	–	–
Fats and oils	+	+	+	+
Minerals	+	+	+	+
Vitamins and trace elements	+	+	+	+

[1] B&K Universal AB, Sollentuna, Sweden.
[2] SDS AB, London, UK.
+with.
–without.

and resuspended in PBS to a final concentration of 10^9 colony-forming units (cfu)/ml (2).

Experimental ***Design.*** Experiment *1:* Mice were fed the four different diets for seven days before infection. The animals were infected orally through a feeding tube (Outer diam = 0.1 cm) three times at 2-day intervals with 0.1 ml of a bacterial suspension containing 10^9 cfu/ml (31). Uninfected control animals were given phosphate-buffered saline instead of the bacterial suspension. For each diet, 10 mice were infected with *H.* pylori and *6* mice served as controls, one half of each group of mice was sacrificed at 2 and 4 weeks post inoculation.

Experiment 2: Forty mice were infected the same way as above and fed *RM2.* After 8 weeks of infection, they were divided into 4 groups. Group 1 was kept on RM2 diet; group 2 changed to *RMI* diet, group 3 changed to *RMA* diet, and group 4 changed to SDS diet. Mice were fed the new diet for 10 days and sacrificed.

Mice were killed by carbon dioxide and stomachs were collected. Each stomach was opened through the longer curvature using sterile surgical instruments. One half of the stomach and duodenum, covering all subtypes of mucosa, was used for pathology. The remaining part of the stomach was used for culture and PCR.

Culture. One half of the entire gastric mucosa was scraped off and homogenised with 500 µl PBS. 100 µl of homogenate was inoculated on GAB-Camp agar and incubated for 7 days under microaerobic conditions at 37°C. The presence of *H.* pylori on the culture plates was confirmed by urease, catalase and oxidase tests, Gram staining and PCR analysis with a urease primer (21).

DNA extraction and PCR conditions. DNA was extracted from frozen homogenates of stomach biopsies from uninfected and *H.* pylori-infected mice (17).The DNA

pellet was dissolved in 50 μl of water and stored at −20 °C until the PCR was completed. HPU primers (Scandinavian Gene Synthesis, Koping, Sweden) based on a urease A gene sequence of *H. pylori* were used and PCR conditions were according to *Nilsson* et al. (21).

Histopathology. Stomach specimens were fixed in 10% buffered formalin (effective osmolar pressure 300 mosm/l and embedded in paraffin. 4 μm sections were prepared and stained with haematoxylin and eosin following standard procedures. Three areas of the stomach were examined: body, antrum and duoduenum. The degree of inflammation in these different parts was scored on a 0–3 scale (31).

Statistical Analysis. The chi-square test was used for analysis of culture and PCR results. The Mann-Whitney *U* test was used for defining inflammation distribution. The level of significance chosen was $p < 0.05$.

Results

Experiment 1: Among the mice fed the RM2, 10 out of 10 exhibited identified *H. pylori* colonies whereas the RMA, RM1 and SDS diets resulted in shares of 20%, 10% and 10%, respectively, of identified *H. pylori* colonies upon culture from the stomach at 2 and 4 weeks post infection (Table 2). A significant difference ($p < 0.01$) was found to exist between the RM2 diet and the other diets.

Table 2. Culture of stomach biopsies at 2 and 4 weeks post inoculation (experiment 1). The number of culture-positive animals when the groups were having one diet throughout the experiment. Five animals were sacrificed at each time point.

Diets/Time point (No. of culture-positive mice/Total No. of mice examined)	2w. post infection		4w. post infection		Total H. *pylori* infection %
	Non-infected	infected	Non-infected	infected	
Standard No. 1 diet (RM1)	0/3	1/5	0/3	0/5	10
Standard No. 2 diet (RM2)	0/3	5/5*	0/3	5/5*	100
Autoclavable diet (RMA)	0/3	1/5	0/3	1/5	20
Standard diet (SDS)	0/3	0/5	0/3	1/5	10

* $p < 0.01$ compared to all other diets. The colonies of H. *pylori* on the GAB-Camp were identified by Gram stain, urease, catalase, oxidase test and PCR.

Experiment 2: The control animals sacrificed (n = 4) before the change of diets were all colonized and *H*. pylori could be cultured from their stomachs. After eight weeks, the diets were changed and mice were sacrificed after an additional 10 days. Mice fed the RM2 diet gave the highest *H*. pylori recovery rate among the four different diets (90%) whereas RMA, RM1 and SDS diets gave 60, 50 and 50% recovery rates, respectively (Table 3).

PCR signals of murine gastric biopsies are shown in Fig. 1. The signals showed a good correlation with the number of bacteria growing on GAB-Camp agar. All non-infected mice were negative in PCR analysis. We found 26 infected mice negative in culture but positive in the PCR (Table 4).

The inflammatory infiltrate comprised mainly granular cells scored as follows: 0, normal tissue; 1, few inflammatory cells; 2, moderate inflammatory cells in several layers; 3, high level of inflammation with foci containing > 50 inflammatory cells, often more than three cell layers deep (Fig. 2). There were no significant differences in histological changes between the non-infected control animals fed the different diets. All *H*. pylori-infected mice produced a significantly higher degree of inflammation than did the non-infected control mice respectively ($p < 0.05$). Infected mice receiving RM2 diet showed the highest inflammation score among these four different commercial diets. This was, however, not significant according to the Mann-Whitney U-test (Fig. 3).

Discussion

Our data indicate that the mouse diet influences culture results of *H*. pylori from infected BALB/cA mice. The changes were most dramatic when the infection was carried out on animals already on a specific diet (Table 2). There were less pronounced differences when the animals were first infected on one diet (RM2) and eight weeks later changed to other diets (Table 3). These data suggest that the most crucial time was when the animals were infected. Once the *H*. pylori infection has started it better withstands the other diets. In other studies, we have seen the same effects of diets on other mouse strains (C57BL/6, NMRI, CBA/Ca and BALB/cJ mice) in that RM2 diet allows us to

Table 3. Culture of stomach biopsies at 10 weeks post inoculation (experiment 2). The number of culture-positive animals at 10 weeks and a change of diet at 8 weeks after infection. All mice were sacrificed at the same time point

Groups	*H. pylori* infection on culture
RM2 diet as control	9/10
RM2 changed to RM1	5/10
RM2 changed to RMA	6/10
RM2 changed to SDS	5/10

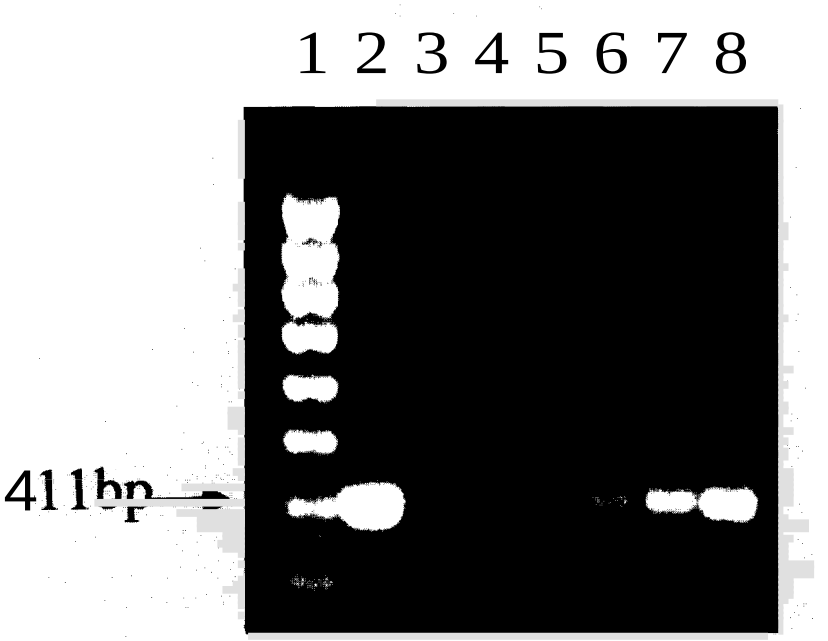


Fig. 1. PCR analysis of *H.* pylori in murine gastric biopsy samples. Lane 1, DNA ladder; Lane 2, *H. pylori*-positive control; Lane 3, water control; Lane 4 and 5, gastric biopsy samples from non-infected mice; Lane 6, gastric biopsy samples from culture-negative infected mouse with RMA diet; Lane 7, gastric biopsy samples from culture-positive infected mouse with RMA diet; Lane 8, gastric biopsy samples from infected mouse with RM2 diet.

Table 4. PCR analysis of mice stomachs from experiments 1 and 2.
Experiments 1 and 2 taken together and analyzed by PCR. When the results from culture and PCR-positive and culture-negative but PCR-positive are added together, the total number of infected animals is obtained

Diets / Groups (No. of mice)	Non-infected		Infected		*H.* pylori infection
	Culture –/PCR–	Culture +/PCR+	Culture –/PCR+	Culture –/PCR–	
RM1	**6**	**6** (30%)	8 (40%)	**6** (30%)	70%
RM2	**6**	19 (95%)	1 (5%)	*0*	100%
RMA	***6***	8 (40%)	8 (40%)	4 (20%)	80%
SDS	**6**	**6** *(30%)*	9 **(45%)**	*5* (25%)	75%

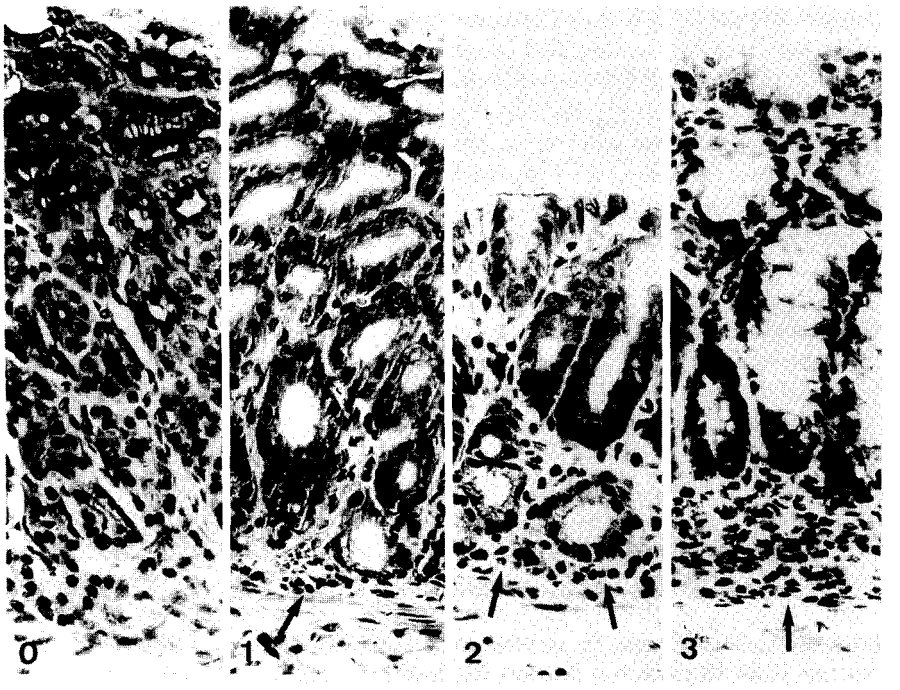


Fig. 2. Histopathological analysis of mice stomachs. The inflammation were scored between 0–3. 0, normal tissue; **1,** few inflammatory cells; **2,** moderate inflammatory cells in several layers; **3,** high level of inflammatory cells, often more than three cell layers deep.

culture *H. pylori* from stomach biopsies whereas **SDS** diet does not (data not shown). We could not observe any direct toxicity of the diets for *H. pylori* in vitro with different diets solubilized in PBS, soaked filter papers in the mix and added them to newly subcultured *H. pylori*. An absolutely sterile diet is not needed since we have seen the same infection rates irrespective of whether using a irradiated sterile RM2 and the conventional RM2.

The mice fed RM2 diet gave the "best" *H. pylori* recovery rate and also the highest inflammation score among the four commercial diets (Fig. **3),** indicating that a higher density of *H. pylori* causes a more severe inflammation.

PCR analysis did not reveal false negative animals when compared to culture results i. e. all culture positive animals were also PCR-positive. However, the number of culture-negative but PCR-positive animals has shown that PCR in this case is superior to culture to determine the presence of *H. pylori* (Table **4) .**The PCR-positive signals from mice with a non-culturable number of *H. pylori* might suggest that a fairly large number of *H. pylori* (more than 1000 bacteria) was necessary to get positive culture results, or that

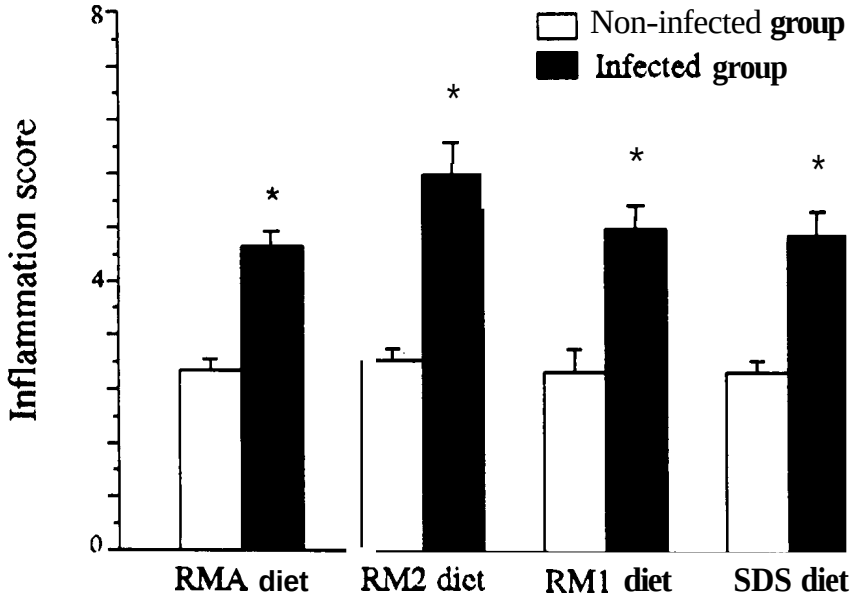


Fig. 3. Inflammation score in the stomach: *H. pylori* infection in BALB/cA mice with different diets. $^{*}P < 0.05$ **vs.** non-infected group.

these bacteria seen in the PCR analysis were present as the non-culturable coccoid form.

The four diets examined in this investigation had few differences in the contents or amounts of the different compounds. Table 1 summarizes these differences, the lack of fish meal and vitamin B12 might be part of the reasons why RM2 sustains *H. pylori* better than the other three diets. Another aspect might be the production process where RM2 is heat-treated at 140–150"C before the pellets are made. If it is the heat treatment that makes the difference, diets have to be heated to at least 110°C since the autoclavable diet is heated to 105"C for 20 minutes. The heat (140–150"C) might destroy components or boil away substances with a low boiling point that have detrimental effects on *H. pylori.* There is approximately the double amount of Soya bean meal in RM2 and RMA than in RM1 and SDS diets (24 and 20.8% compared to 14 and 10.7%, respectively). Interestingly, *Tsugane* et al. showed an increased risk of atrophic gastritis for individuals with a high intake of soybean products (29). The use of fish is very common in animal diets, being cheap and easily accessible for the producers. Vitamin E-deficient diets enriched with fish oil were found to suppress lethal *Plasmodium yoelii* infections in athymic mice. This is one out of many positive effects fish products can have **(27).** The amounts of vitamin A in the 4 diets were similar and cannot be responsible for the differences.

The conclusion will end up with 3 candidates (no fish products, high temperature and high amounts of Soya products) being able to increase the ability of *H.* pylori to grow in BALB/cA mice. However we were unable to pinpoint the exact causes of the phenomenon reported.

Lee et al. (19) recently reported on the importance of standardizing the mouse model and to use a well-defined mouse passage strain of *H. pylori.* However, many other investigations who developed animal models to study *H.* felis and *H.* pylori infections ignored the importance of defining the diet(s) used. The value of optimizing the diet to allow a maximal "take" and a high inflammation score for clinical isolates as well as mouse-adapted strains is very important. We will now explore alternative diets, so called proxidants which may dramatically affect the outcome of experimental studies of possible protective effects of vitamins and other antioxidants in our mouse model. Recent studies by Sandersson et al. **(24)** and *Zhang* et al. **(34)** suggested that antioxidants might play a very important role in protecting the gastric mucosa against *H.* pylori-induced free radicals, their effects on cell and DNA damage and later development of gastric carcinomas. The high number and/or high quantity of some dietary components in the food may be attributed to the detoxification of carcinogens and the scavenging reactive carcinogen species to prevent the development of cancer **(23).**

Further studies will elucidate whether specific component(s) is/are responsible for the decreased ability to recover *H.* pylori from infected mice.

Acknowledgements. We would like to express our gratitude towards Jan Strid, BK Universal AB, Sweden, for providing food samples to this study. This study was supported by a grant from the CN 70 Foundation in Stockholm, Forssmans Foundation and Swedish Medical Research Council 16 X 04723.

References

1. *Aldoori, W. H., E. L. Giovannucci, M. J. Stampfer, E. B. Rimm, A. L. Wing,* and *W. C. Willett:* Prospective study of diet and the risk of duodenal ulcer in men. Am. J. Epidemiol. 145 (1997) 42–50
2. *Aleljung, P.,* H. *O. Nilsson, X. Wang, P. Nyberg, T. Mörner, I. Warsame,* and *T. Wadstrom:* Gastrointestinal colonisation of BALB/c **A** mice by *Helicobacter pylori* monitored by heparin magnetic separation. FEMS Immuno. Med. Microbiol. **13** (1996) 303–309
3. *Alexander, J. W.:* Immunoenhancement via enteral nutrition. Arch. Surg. 128 (1993) 1242–1245
4. *Chang, H. R., A. G. Dulloo, I. R. Vladoianu, P. F. Piguet, D. Arsenijevic, L. Girardier,* and *J. C. Pechere:* Fish oil decreases natural resistance of mice to infection with *Salmonella typhimurium.* Metabolism. 1 (1992) 1–2
5. *Correa, P.:* Diet modification and gastric cancer prevention. Natl. Cancer Inst. Monogr. 12 (1992) 75–788
6. *Erskine, R. J.:* Nutrition and mastitis. Vet. Clin. North Am. Food Anim. Pract. 9 (1993) 551–561

7. *The Eurogast Study Group:* An international association between *Helicobacter pylori* infection and gastric cancer. Lancet 341 (1993) 1359–1362
8. *Fevang, P., H. Saav,* and *A. T. Hostmark:* Dietary fish oils and long term malaria protection. Lipids 30 (1995) 437–441
9. *Fritsche, K. L., L. M. Shahbazian, C. Feng,* and *N. Berg:* Dietary fish oil reduces survival and impairs bacterial clearence in C3H/Hen mice challenged with *Listeria monocytogenes.* Clin. Sci. 92 (1997) 95–101
10. *Gennari, R., J. W. Alexander,* and *T. Eaves-Pyles:* Effect of different combinations of dietary additives on bacterial translocation and survival in gut derived sepsis. Jpn. J. Parenter. Enteral. Nutr. 19 (1995) 319–325
11. *Hansson, L. E., O. Nyrén, W. Hsing, R. Bergstrom, S. Josefsson, W. H. Chow, J. E Fraumeni,* and *H. O. Adami:* The risk of stomach cancer in patients with gastric or duodenal ulcer disease. N. Engl. J. Med. 335 (1996) 242–9
12. *Harbridge, L. S.:* Nutrition and immunity with emphasis on infection and autoimmune disease. Nutr. Health 10 (1996) 285–312
13. *Hazell, S. L.* and *D. Y. Graham:* Unsaturated fatty acids and viability of *Helicobacter (Campylobacter) pylori.* J. Clin. Microbiol. 5 (1990) 1060–1061
14. *Hvang, H., J. Dwyer,* and *R. M. Russel:* Diet, *Helicobacter pylori* infection, food preservation and gastric cancer risk: are there new roles for preventative factors? Nutr. Rev. 52 (1994) 75–83
15. *International Agency for Research on Cancer:* IARC Monographs on the evaluation of carcinogenic risks to humans. Vol 61. Lyon: World Health Organisation, 177–240 (1994)
16. *Kanke, Y., Y. Iitoi, M. Iwasaki, Y. Iwase, M. Iwama, M. Kimira, T. Takahashi, S. Tsugane, S. Watanabe,* and *Akabane:* Effects of human diets of two different Japanese populations on cancer incidence in rat hepatic drug-metabolizing and antioxidant enzyme systems. Nutr. Cancer 26 (1996) 63–71
17. *Kong, L., J. G. Smith, D. Bramhill, G. K. Abruzzo, C. Bonfiglio, C. Cioffe, A. M. Flattery, C. J. Gill, L. Lynch, P. M. Scott, L. Silver, C. Thompson, H. Kropp,* and *K. Bartizal:* A sensitive and specific PCR method to detect *Helicobacter felis* in a conventional mouse model. Clin. Diagn. Lab. Immunol. 1 (1996) 73–78
18. *Lacey, C. J., M. E. Murphy, M. J. Sanderson, E. F. Monteiro, A. Vail,* and *C. J. Schorah:* Antioxidant-micronutrients and HIV infection. Int. J. STD. AIDS. 7 (1996) 485–489
19. *Lee, A., J. O'Rourke, M. C. D. Ungria, B. Robertson, G. Daskalopoulos,* and *M. F. Dixon:* A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterol. 112 (1997) 1386–1397
20. *Niles, R. M.:* Use of vitamines C and D in chemoprevention and therapy of cancer: control of nuclear receptor expression and function. Vitamins, cancer and receptors. Adv. Exp. Med. Biol. 375 (1995) 1–15
21. *Nilsson, H. O., P. Aleljung, I. Nilsson, T. Tyszkiewicz,* and *T. Wadstrom:* Immunomagnetic bead enrichment and PCR for detection of *Helicobacter pylori* in human stools. J. Microbiol. Methods 27 (1996) 73–79
22. *Phull, P. S., C. J. Green,* and *M. R. Jacyna:* A radical view of the stomach: the role of oxygen-derived free radicals and anti-oxidants in gastroduodenal disease. Eur. J. Gastroenterol. Hepatol. 7 (1995) 265–274
23. *Ramarathnam, N., T. Osaw, H. Ochi,* and *S. Kawakishi:* The contribution of plant food antioxidants to human health. Trend Food Sci. Tech. 6 (1995) 75–82

24. *Sanderson, M.J., K.L.M. White, I.M. Drake,* and *C.J. Schorah:* Vitamin E and carotenoids in gastric biopsies: the relation to plasma concentrations in patients with and without *Helicobacter pylori* gastritis. Am. J. Clin. Nutr. 65 (1997)101–106
25. *Schloss, I., M.S. Kidd, H.Y. Tichelaar, G.O. Young,* and *S.J. O'Keefe:* Dietary factors with a low risk of colon cancer in coloured west coast fishermen. S. Afr. Med. J. 87 (1997)152–158
26. *Soltez, V., C. Schalén,* and *P.A. Mdrdh:* New selective for *Campylobacter pylori*. In: Kaijser B, Falsen E (eds) Proceedings of the Fourth International Workshop on *Campylobacter* infections. University of Gothenburg, Gothenburg, Sweden. 433–436 (1988)
27. *Taylor, D.W., O.A. Lecander, V.R. Krishna, C.B. Evans, V.C. Morris,* and *J.R. Barta:* Vitamin E-dificient diets enriched with fish oil suppress lethal Plasmodium yoelii infection in athymic and scid/bg mice. Infect. Immun. 65 (1997) 197–202
28. *Thompson, L., A. Cockayne,* and *R.C. Spiller:* Inhibitory effect of polyunsaturated fatty acids on the growth of *Helicobacter pylori:* a possible explanation of the effect of diet on peptic ulceration. Gut 35 (1994)1557–1561
29. *Tsugane,* S., *M. Kabuto, H. Imai, F. Gey, Y. Tei, T. Hanaoka, K. Sugano,* and *S. Watanabe:* Helicobacter pylori, dietary factors, and atrophic gastritis in five Japanese populations with different gastric cancer mortality. Cancer Causes Control. 4 (1993)297–305
30. *Tsugane,* S., *Y. Tei, T. Takahashi, S. Watanabe,* and *K. Sugano:* Salty food intake and risk of *Helicobacter pylori* infection. Jpn. J. Cancer Res. 85 (1994)474–478
31. *Wang, X., E. Sturegdrd, R. Rupar, H.O. Nilsson, P. Aleljung, B. Carlén, R. Willén,* and *T. Wadstrom:* Infection of BALB/c *A* mice by spiral and coccoid forms of *Helicobacter pylori*. J. Med. Microbiol. 46 (1997)657–663
32. *Werner,* G.H. and *P. Jolles:* Immunostimulating Agents: what next? *A* review of their present and potential medical applications. Eur. J. Biochem. 242 (1996)1–19
33. *Wong, R.M., Y. Kondo, H. Banba, S. Matsuzuki,* and *S. Sekine: Anti-Helicobacter pylori* activity in the garlic extract. Nipp. Shokaki Gakk. Zasshi 9 (1996)688
34. *Zhang,* O., *J.B. Dawodu, G. Etolhi, A. Husain, C.G. Gemmell,* and *R.I. Russell:* Relationship between the mucosal production of reactive oxygen radicals and desity of *Helicobacter pylori* in patients with duodenal ulcer. Eur. J. Gastroenterol. Hepatol. 9 (1997)261–265

Corresponding author: *Torkel Wadstrom* MD, PhD, Professor, Department of Medical Microbiology, Lund University, Solvegatan 23, S-22362 Lund, Sweden, Fax: +4646 15 25 64, E-mail: tw-lab@mmb.lu.se

III

RAPD-PCR, Histopathological and Serological Analysis of Four Mouse Strains Infected with Multiple Strains of *Helicobacter pylori*

Xin Wang', Roger Willén[2], Torkel Wadstrom' and Par Aleljung'

From the 'Department of Infectious Diseases and Medical Microbiology, *Department of Pathology, University of Lund, Lund, Sweden

Correspondence to: Torkel Wadstrom, MD, PhD, Department of Infectious Disease and Medical Microbiology, University of Lund, Solvegatan 23, S-223 62 Lund, Sweden. Tel: +46 46 17 32 40; Fax: +46 46 15 25 64; E-mail: Torkel.Wadstrom@alinks.se

Microbial Ecology in Health and Disease 1998; 10: 148–154

The aims of this study were to investigate: i) if a specific strain(s) became predominant in the stomach of mice infected with multiple strains of freshly isolated *H. pylori*; ii) if *a* time dependent selection of strains occurs; iii) to compare the degree of gastric inflammation in C57BL/6, CBA/Ca, BALB/cA and NMRI mice: and iv) to follow the serological response to the infection. Six weeks old mice were inoculated orally 3 times at 2-day intervals with 9 freshly isolated *H. pylori* strains. Five animals of each mouse strain were sacrificed at 2, 4, 10 and 16 weeks post-inoculation. Stomach smears were cultured on GAB-Camp agar at 37°C for 5–10 days under microaerophilic conditions. Seven to 10 single *H. pylori* colonies from each animal were subcultured and identified by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis. Histopathology of the gastric mucosa and serum antibody response were also analysed for each animal.

Most mice were found to be infected by 1 strain (119/95). In only a few animals were multiple strains recovered. Strain 119/95 constituted 90.5% of the 577 mouse-passaged isolates and when tested it expressed both vacuolating cytotoxin antigen (VacA) and cytotoxin associated antigen (CagA). The inflammation scores of murine stomachs and duodenum samples of infected animals were significantly higher than in non-infected control animals for all 4 mouse strains. No significant differences in inflammation score were observed for the mouse strains 16 weeks post-infection. The immune response was also higher in infected versus non-infected animals for all mouse strains. The specific serum anti *H. pylori* immune response was highest for the NMRI mice followed by BALB/cA, C57BL/6, and CBA/Ca mice. *Key words*: *Helicobater pylori*, gastritis, RAPD-PCR, C57BL/6, CBA/Ca, BALB/cA, NMRI, mice, histopathology. serology

INTRODUCTION

Helicobacter pylori causes chronic active type B gastritis in humans and is associated with the development of peptic ulcer disease and gastric cancer (1–3). In the last few years animal models have been developed to study the pathogenesis of *H. pylori* infection, in gnotobiotic piglets, mice, mongolian gerbils and primates (4–9).

Multiple strains of *H. pylori* may infect a person during long periods of time (10). A monkey model was used to infect animals with multiple strains of *H. pylori* (11). The observation that *H. pylori* strains can cause a varying degree of inflammation in BALB/cA mice (9) and by utilising multiple strains to infect mice, makes it possible to investigate if strains can act synergistically and become more detrimental for the host. RAPD-PCR (random amplified polymorphic DNA-polymerase chain reaction) analysis has been used successfully for typing *H. pylori* (12, 13) and found to be a good method to identify the original and re-isolated strains from infected animals. Different strains of *H. felis* and *H. pylori* cause moderate to severe gastritis in mice and results seem to vary between laboratories with conflicting results (14–17). Certain criteria were established to overcome some of these difficulties in Lausanne (1995) (18). It seems that some of these difficulties are overcome by using the Sydney strain capable of infecting a variety of mouse strains though the degree of inflammation varies in different mice strains (17, 19).

Most investigations have focused on Type I strains (CagA and VacA producing) and their ability to infect but have also been able to show the inability of the Type II strains to colonise. However, Kimura et al. have shown that Type II (CagA and VacA negative) strains colonise equally well but the strain tested did not cause inflammation in a hairless mouse model (16).

To further characterise this model for *H. pylori* type B gastritis we investigated: i) which strain(s) became the predominant in murine stomachs infected with a mixture of 9 freshly isolated strains, ii) whether there would be changes in the composition of strains with time, iii) the

 ISSN 0891-060X

Table I

Origin of H. *pylori strains and CagA and VacA expression*

Strains	Clinical manifestation	CagA	VacA
553/93	Gastritis	–	–
83 95	Duodenal ulcer	+	–
87/95	Duodenal ulcer	+	+
105 95	Atrophic gastritis	+	+
116/95	Gastritis	+	+
119/95	Duodenal ulcer	+	+
122/96	Duodenal ulcer	+	+
125196	Pyloric ulcer	+	+
131'96	Gastritis	+	+

degree of inflammation scores and iv) immune response to *H. pylori* infection in C57BL/6, CBA/Ca, BALB/cA and NMRI mice.

MATERIALS AND METHODS

Bacterial strains

H. pylori strains 553/93, 83/95, 87/95, 105/95, 116/95, 119/95, 122/96, 125196 and 131/96 were fresh isolates (frozen once after isolation) from human stomach biopsies (Table I). All 9 strains were grown on GAB-Camp agar for 48 h at 37°C under microaerophilic conditions (20) and subcultured twice. Only 1 colony was transferred to a new agar medium to ensure 1 clone per strain. Each strain was harvested and suspended in PBS, diluted to 10^9 colony-forming units/ml (cfu/ml) of mainly the spiral shaped form (85–95%), equivalent to a defined absorbency value ($A_{540nm} = 1.0$) and mixed with the other strains in equal amounts. Methods for the determination of CagA and VacA expression, SDS-PAGE and subsequent immunoblot (IB) analysis were performed as described by Nilsson et al. (21).

Animals

Six-week-old C57BL/6 (n = 20), CBA/Ca (n = 20), BALB/cA (n = 20) and NMRI (n = 15) mice were used in this study (B&K Universal Company, Sollentuna, Sweden). The animals were housed with a 12 h light–dark schedule and fed a commercial rodent diet no. 2 (RM2, B&K Universal Company, Sollentuna, Sweden) and water *ad libitum* (22).

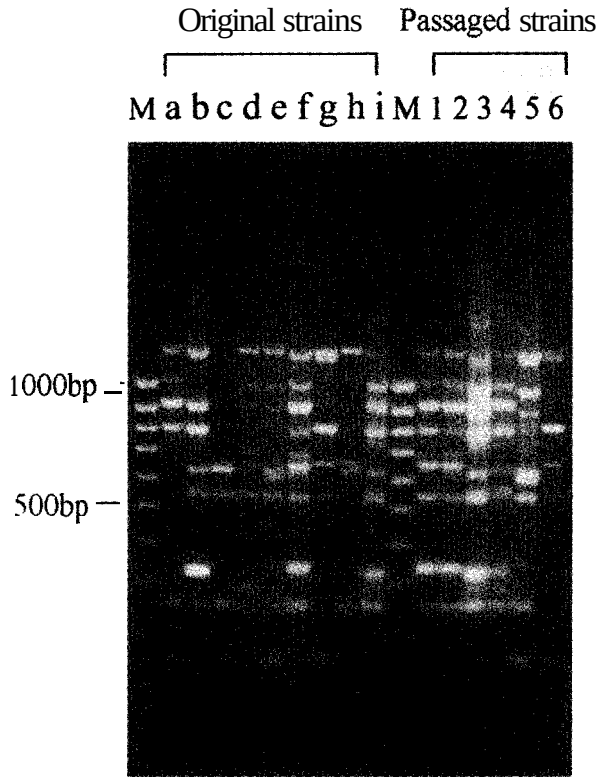


Fig. 1. DNA amplification patterns of 9 *H. pylori* strains. Lane M, DNA ladder from 1000 bp to 100 bp; Lanes a–i, original strains 553/93 (a), 83/95 (b), 871958 (c), 105/95 (d). 116/95 (e). 119/95 (f), 122/96 (g), 125/96 (h) and 131/96 (i); Lanes 1 and 2, passaged strains from a NMRI mouse 10 weeks p.i. and a BALB/cA mouse 16 weeks p.i., respectively, are identical to Lane f; Lanes 3 and 4, passaged strains from a BALB/cA mouse 4 weeks p.i. and a C57BL/6 mouse 16 weeks p.i. are identical to Lane i; Lane 5, passaged strain from a C57BL/6 mouse 2 weeks p.i. is identical to Lane e: Lane 6. passaged strain from a CBA/Ca mouse 2 weeks p.i. is identical to Lane g.

Table II

Number of mice colonised by 1, 2 and 3 RAPD-types of H. pylori strains

Mouse strain/*H. pylori* strains	2 weeks p i			4 weeks p.1.			10 weeks p.1			16 weeks p 1.		
	1	2	3	1	2	3	1	2	3	1	2	3
C57BL/6	3	2	0	5	0	0	5	0	0	4	1	0
CBA/Ca	2	2	1	5	0	0	5	0	0	5	0	0
BALB/cA	3	2	0	4	1	0	5	0	0	5	0	0
NMRI	5	0	0		NT		5	0	0	5	0	0

p.i. = Post inoculation.
NT = not tested.

Experimental design

Mice were inoculated orally through a feeding tube (outer diameter = 1.3 mm) 3 times at 2-day intervals with 0.1 ml of a mixture of 9 *H. pylori* strains in suspension (10^9 cfu/ml). PBS was used as a negative control (9). The bacteria were cultured freshly on agar prior to infection. Five of each mouse strain were sacrificed with CO, at 2, 4, 10 and 16 weeks post-inoculation (except 4 weeks post-inoculation of NMRI mice). Blood and stomach biopsies were subsequently collected. Murine stomachs were opened through the longer curvature using sterile surgical instruments. One half of the stomach and duodenum, covering all subtypes of mucosa, was subjected to histopathology analysis and the remaining part was used for culture.

Culture

Half of the stomach biopsy was rinsed in PBS and smeared (rubbed) directly on GAB-camp agar and incubated at 37°C for 5–10 days in microaerophilic conditions. From each mouse, 7–10 single colonies were isolated, subcultured for DNA extraction and analysed by RAPD-PCR.

DNA extraction and PCR conditions

Individual colonies from GAB-Camp agar were isolated using sterile toothpicks and suspended in 50 µl of 10 mM EDTA, pH 8.0, in eppendorf tubes. Freshly prepared cracking 2X buffer (50 µl, 0.67 M NaOH, 10% SDS, 20% sucrose) was added and mixed by vortex. All tubes were incubated at 70°C for 5 min and 1.5 µl of 4M KCI added after the tubes were cooled to room temperature. Samples were then put on ice for 5 min and centrifuged at 12000 *g* for 3 min at 4°C. DNA was precipitated by adding 1/10 volume of 4M LiCl to the supernatant and 2.5 volumes of absolute ethanol incubated at −20°C for 20 min. DNA was pelleted by centrifugation at 12000 *g* for 10 min, rinsed by 70% ethanol and suspended in 50 µl PCR grade water.

A mixture of two primers were used in this study: 1254 and 1283 (23). A conventional PCR-buffer was used for amplification; consisting of 10 mM Tris–HCl (pH 8.3). 50 mM KCl, 3 mM $MgCl_2$, 0.01% BSA, 0.2 mM of each deoxynucleotide-triphosphosphate, 0.8 µM of each primer and Taq polymerase (1.875 U; Boehringer Mannheim, Mannheim, Germany). PCR condition consisted of 5 cycles of 5 min at 94°C, 5 min at 36°C and 5 min at 72°C followed by 30 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C with a final elongation step of 10 min at 72°C. Amplified products (15 µl) were analysed by agarose electrophoresis (1.5% gels) and visualised by ethidium bromide staining. DNA amplification patterns were considered identical when no variation was observed in the DNA banding patterns.

Histopathology

Murine stomach tissues were fixed in 10% buffered formalin (effective osmolar pressure 300 mosm/l) and embedded in paraffin. Sections of 4 µm were prepared and stained with haematoxylin and eosin by standard procedures. The degree of inflammation was scored (0–3) 'blindly' for body, antrum and duodenum by one pathologist: 0 = normal: 1 = few inflammatory cells; 2 = moderate amounts of inflammatory cells in several layers; 3 = high level of inflammation with nests containing more than 50 inflammatory cells, often more than three cell layers (9).

Immunoblot

Antigen preparation, performance of SDS-PAGE and subsequent immunoblot (IB) analysis were performed as described by Nilsson et al. (21). The term specific antibody response is used when antisera of infected animals recognises blotted proteins whereas the non-infected controls does not.

Statistical Analysis

The Mann–Whitney *U* test was used to determine inflammation distributions. The level of significance selected was $p < 0.05$.

RESULTS

All *H. pylori* infected animals gave positive stomach culture results whereas none of the non-infected control mice were *H. pylori* culture positive. The number of colonies on GAB-Camp agar cultured from stomach biopsies of in-

Table III

Recovery of a predominant H. pylori strain in 4 mouse strains (within parentheses = number of strains versus tested re-isolated strains)

Number of HP strains (% in each strain of mice)	Strain119/95	Strain 131/95	Strain 116 95	Strain 122/96	Total tested strains
C57BL/6	158 (88.8)	18 (10.1)	2 (1.1)	0	178
CBA/Ca	125 (87.4)	15 (10.5)	0	3 (2.1)	143
BALB cA	138 (89.0)	16 (10.3)	1 (0.7)	0	155
NMRI	101 (100)	0	0	0	101
Total number of strains	522 (90.5)	49 (8.5)	3 (0.5)	3 (0.5)	577

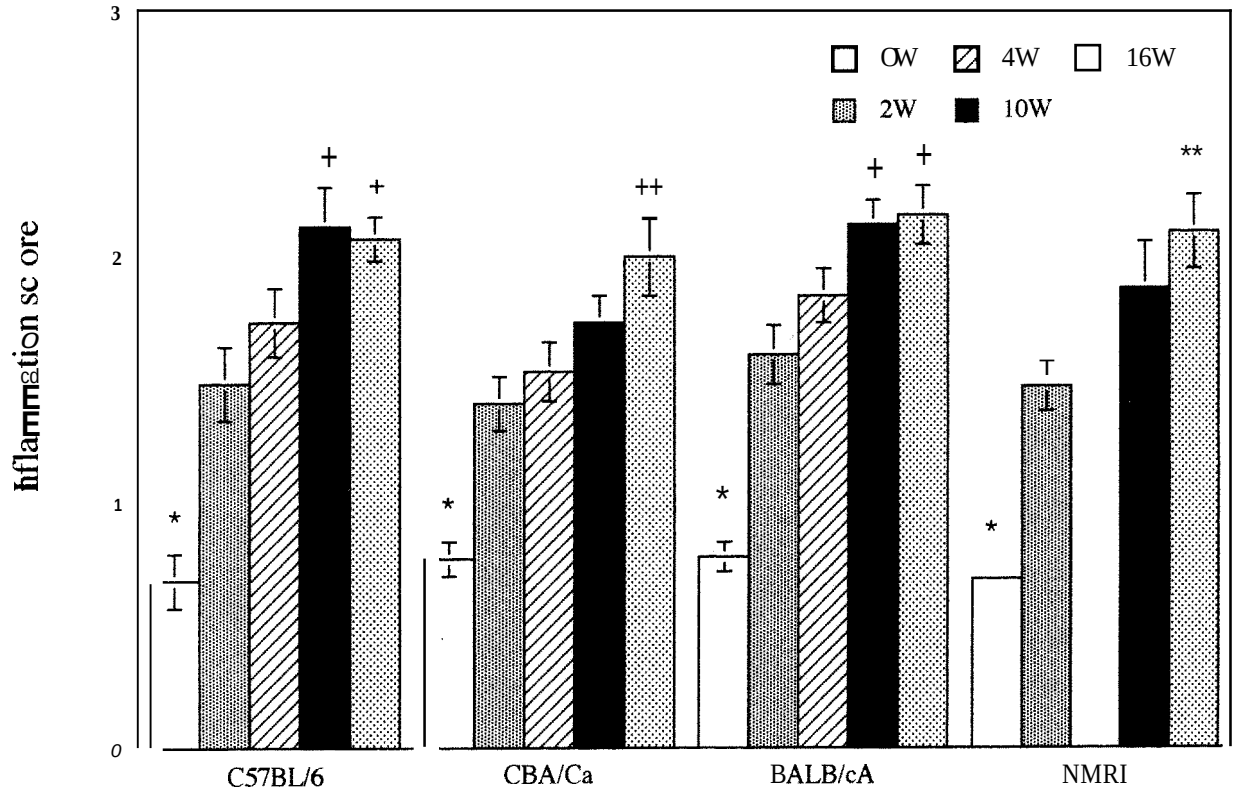


Fig. 2. Inflammation score of *H. pylori* infected stomachs of 4 strains of mice. * $p < 0.01$ non-infected normal *vs.* all infected animals, + $p < 0.05$ *vs.* 2 week and 4 week p.i. in C57BL/6 and BALB/cA mice, ++ $p < 0.05$ *vs.* 2 week and 4 week p.i. in CBA/Ca mice, ** $p < 0.01$ *vs.* 2 week p.i. in NMRI mice.

fected animals showed no significant difference between the 4 mouse strains studied (data not shown).

A total of 577 single colonies of *H. pylori* were collected from culture positive animals. All *H. pylori* strains isolated from infected mice were identified as one of the original inoculated strains. Table I1 shows the number of mice colonised by 1, 2 or 3 *H. pylori* strains following infection. One dominant strain colonised 66 of 75 animals (88%), whereas 8 (10.7%) had 2 strains of *H. pylori* and only 1 animal (1.3%) was found with 3 strains in its stomach. Of the mice (n = 9) with 2 or 3 strains, 7 (78%) of them were recovered at 2 weeks post-inoculation and the other two mice were found at 4 and 16 weeks post-infection.

RAPD patterns of the 9 *H. pylori* strains originally used to infect all 4 mouse strains and the 6 strains re-isolated from infected animals are shown in Fig. 1. Nine original strains (553/93, 83/95, 87/95, 105195, 116/95, 119/95, 122/96, 125/96 and 131/96) were identified as 9 individual fingerprints using 2 primers: 1254 and 1283 (Fig. 1 a–i). Lane 1 and 2 show two mouse passaged strains from 1 NMRI mouse 10 weeks post-inoculation (p.i.) and BALB/cA mouse 16 weeks p.i. identical to strain 119/95; Lane 3 and 4 show two passaged strains from one BALB/cA mouse 4 weeks p.i. and a C57BL/6 mouse 16 weeks p.i. identical to strain 131196; Lane 5 and 6 show two passaged strains from a C57BL/6 mouse and a CBA/Ca mouse 2 weeks p.i. identical to 116/95 and 122/96, respectively.

Strain 119/95 was the most commonly isolated organism (90.5%) (Table III). Strain 131/96 was recovered from 49 out of 577 tested strains (10.1%), strains 116/95 and 122/96 were recovered 3 times *(0.5%)* and only during the first 2 weeks of the infection in C57BL/6, CBA/Ca and BALB; cA mice. These 4 *H. pylori* strains were all cagA and vacA positive strains (Table I).

Inflammation scores were much higher for *H. pylori* infected animals than non-infected controls for all 4 mouse strains ($p < 0.01$) (Fig. 2). The inflammatory cell infiltration against *H. pylori* were mainly granulocytes during the entire infection in these mice. Significantly higher inflammation scores were obtained after 10 weeks infection compared to 2 and 4 weeks infection in C57BL/6 and BALB/cA mice ($p < 0.05$). NMRI mice showed much higher inflammation scores 16 weeks p.i. compared to 2 weeks p.i. ($p < 0.01$). In CBA/Ca mice, the inflammation scores increased significantly 16 weeks p.i. compared to 2 and 4 weeks p.i. ($p < 0.05$). All 4 mouse strains demonstrated severe gastritis 16 weeks p.i. of *H. pylori* with minor differences between strains (Fig. 3).

A specific immune response to *H. pylori,* analysed by immunoblot, was detected after 4 weeks of infection and

the antibody levels increased in the number and intensity of stained proteins with time (Fig. 4). Antibody levels increased especially against proteins of size 26–29 kDa and 50–60 kDa in the C57BL 6, BALB/cA and NMRI mice. Outbred NMRI mice evoked the strongest immune response in immunoblot. BALB/cA and C57BL/6 mice showed antibody reactivity to the 19, 26–29 and 50–60 kDa proteins whereas CBA/Ca mice showed a weaker response only to the 26–29 kDa and 19 kDa proteins. The 4 mouse strains were ranked in the order of immune response to the *H. pylori* infection starting with the strain that gave the highest number and intensity of stained protein bands in immunoblot were as follows: NMRI > BALB/cA > C57BL/6 > CBA/Ca mice.

DISCUSSION

All 577 *H. pylori* isolates from 75 infected mice of 4 strains were identified as one of the 9 original inoculated bacterial strains; hence, no adaptation mutants were detected. A total of 522 of 577 isolates (90.5%) match the original strain 119195 by RAPD analysis. *H. pylori* strain 119/95 was found to be the most common strain colonising all 4 mouse strains. Strain 119/95 does not seem to have any host restriction within the tested mice and furthermore, this strain has been shown to readily infect guinea pigs (24). Our findings that most mice were infected with one strain and only a few animals were infected with two or three strains which were recovered during the early phase of infection indicate a time dependent decrease of strains which might reflect that strains possess different colonisation abilities of the murine stomach. Dubois et al. (11) investigated isolates from 3 rhesus monkeys infected with 2 human *H. pylori* strains and only one of the strains could be re-isolated. Lee et al. (19) also reported similarly that the Sydney strain (SSI), a *H. pylori* mouse passaged strain, was the only strain to be re-isolated from mice infected by a mixture of 4 fresh clinical isolates. The conclusion could be a simple fact — better colonising ability — but the strains which could not be re-isolated from mice stomachs may not be able to infect mice. Receiving different strains with and without the capacity to infect mice is useful to recognise differences in virulence/colonisation determinants.

The use of Type I strains in animal models dominate and most strains of Type II do not seem to be able to colonise mice. There are exceptions which demonstrate colonisation ability without inflammation **(16)** or inflammation but poor colonisation (5). Further use of type II strains in animal models may resolve ques-

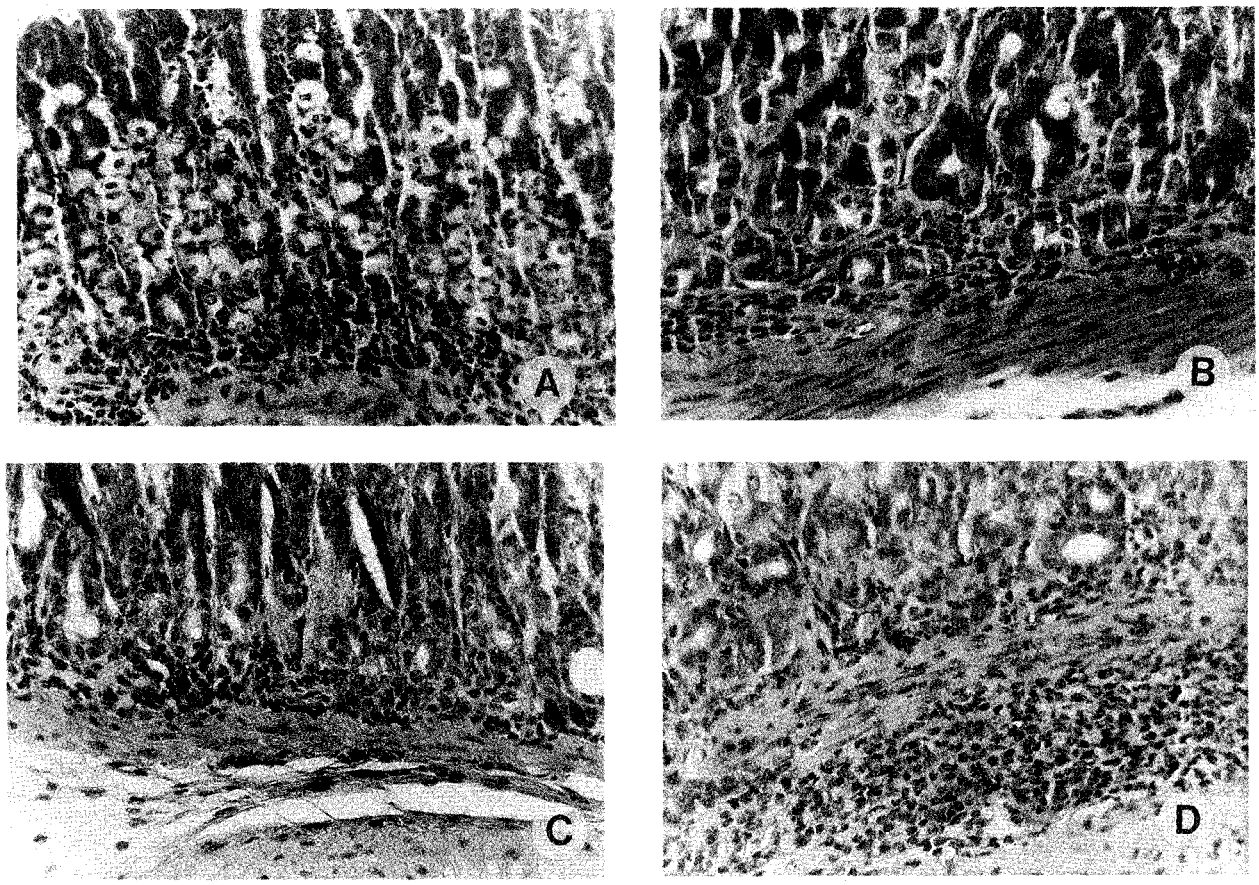


Fig. 3. Histopatological changes of murine stomachs at 16 weeks p.i. of 4 strains of infected mice. **A (BALB/cA** mice), **B (CBA/Ca** mice), C (NMRI mice) and D (C57BL/6 mice) demonstrate a severe (grade 3) inflammatory cell reaction (bottom field). H-E x 150.

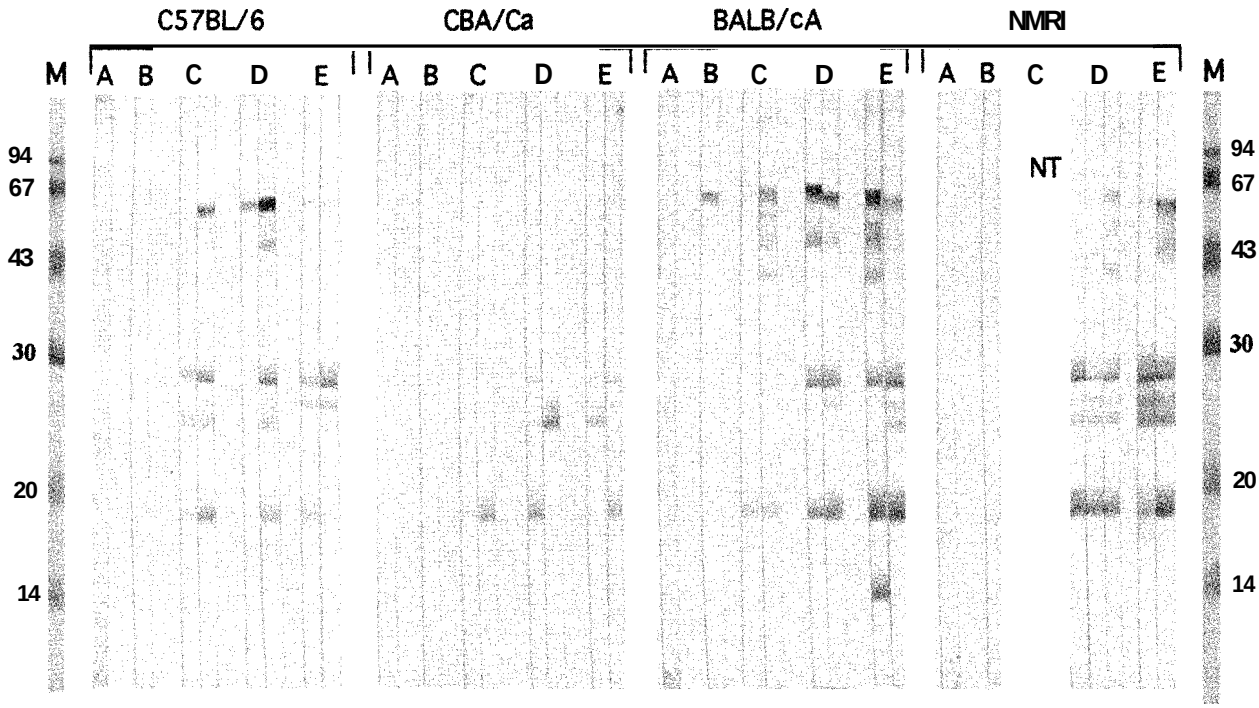


Fig. 4. Immunoblot result of 4 strains of mice. M. standard marker; **A.** normal non-infected mouse: B, *H. pylori* infected mouse 2 weeks p.i.; C, 4 week p.i. mice; D, 10 weeks p.i. mice; E, 16 weeks p.i. mice. NT = not tested in NMRI mice 4 week p.i.

tions regarding the value of these virulence markers in gastric colonisation and inflammation.

In our infection model we observed increased infiltrates of inflammatory cells (mainly granulocytes), within the lower part of the stomach at the gastric and the duodenal mucosa for all three inbred strains used (C57BL/6, BALB/cA and CBA/Ca) as well as for the outbred NMRI strain. CBA/Ca mice gave a significantly lower inflammation score compared to BALB/cA mice at 4 and 10 weeks p.i. but no significant differences in the level of inflammation were observed at 16 weeks p.i. between the 4 mouse strains as analysed by Mann–Whitney *U* test. Thus, the isolate 119/95, which became predominant in mice, does not seem to be host-dependent in the mice investigated. However. Sakagami et al. (25) reported that both C3H/He and C57BL/6 mice exhibited a more severe inflammation by *H. felis* and *H. pylori* infection in these inbred strains of mice. Lee et al. (19) also reported differences in host response in inflammation and colonisation. These conflicting findings concerning host response and host dependence has to be further investigated.

The immune response to *H. pylori* infection could be detected by immunoblot 4 weeks p.i. and increased especially to proteins of the 26–29 kDa region. Outbred NMRI mice showed the best immune response in immunoblot and the immune response was ranked in the inbred mice as follows: BALB/cA > C57BL/6 > CBA/Ca. These differences might be due to the fact that the outbred NMRI mice have a more complete and better functioning immune system. Ferrero et al. (17) observed similar immune response differences between BALB/c and C57BL/6 mice where BALB/c mice acquired much higher antibody titres (EIA) reflecting a better immune response to infection.

Berg et al. ***(26)*** reported that specific *H. pylori* strains colonise monkeys more readily than others and observed differences among different individual animals. These recent observations strongly suggest that development of a well defined experimental model in inbred strains of mice is an attractive strategy to further standardise an experimental infection model to evaluate new prophylaxis and treatment methods for *H. pylori* type B gastritis in humans.

ACKNOWLEDGEMEKTS

This study was supported by grants from the Swedish Medical Research Council (16X04723), the Forssman Foundation in Lund and CN-foundation in Stockholm, Sweden. *H. pylori* strain 119/95 is available upon requests at mailing cost.

REFERENCES

1. Warren JR, Marshal1 B. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 1983; 1273–5.
2. Nedrud JG. Czinn SJ. *Helieohneter pylori.* Curr Opin Gastroenterol 1997: 13: 71–8.

3. Goldstone AR, Quirke P, Dixon MF. *Helicobacter pylori* infection and gastric cancer. J Pathol 1996; 179: 129–37.
4. Engstrand L. Gustavsson S, Jorgensen A, Schwan A. Schevenius **A.** Inoculation of barrier born pigs with *Helicobacter pylori:* a useful animal model for gastritis type B. Infect Immun 1995; 58: 1763–8.
5. Aleljung P, Nilsson HO, Wang X, et al. Gastrointestinal colonisation of BALB/c A mice by *Helicohacter pylori* monitored by heparin magnetic separation. FEMS Immuno Med Microbiol 1996; 13: 303–9.
6. Marchetti M, Arico B, Burroni D, Figura N, Rappuoli R, Ghiara P. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. Science 1995: 267: 1655–8.
7. Matsumoto S, Washizuka Y, Matsumoto Y. et al. Induction of ulceration and severe gastritis in Mongolian gerbil by *Helicobacter pylori* infection. J Med Microbiol 1997; 46: 391–7.
8. Dubois A. Fiala N, Hemanackah NL, et al. Natural gastric infection with *Helicohacter pylori* in monkeys: a model for spiral bacteria infection in humans. Gastroenterol 1994: 106: 1405–17.
9. Wang X, Sturegård E. Rupar R. et al. Infection of BALB/cA mice by spiral and coccoid forms of *Helicobacter pylori.* J Med Microbiol 1997; 46: 657–63.
10. Taylor NS. Fox JG, Akopyants NS, et al. Long-term colonization with single and multiple strains of *Helicobacter pylori* assessed by DNA fingerprinting. J Clin Microbiol 1995; 33: 918–23.
11. Dubois A, Berg DE, Incecik ET, et al. Transient and persistent experimental infection of nonhuman primates with *Helicobacter pylori:* implications for human disease. J Clin Microbiol 1996; 64: 2885–91.
12. Xia HX, Windle HJ. Marshall DG, Smyth CJ, Keane CT, O'Morain CA. Recrudescence of *Helicobacter pylori* after apparently successful eradication: novel application of randomly amplified polymorphic DNA fingerprinting. Gut 1995; 37: 30–4.
13. Marshall DG, Chua A, Keeling PWN, Sullivan DJ, Coleman DC, Smyth CJ. Molecular analysis of *Helicohacter pylori* populations in antral biopsies from individual patients using randomly amplified polymorphic DNA (RAPD) fingerprinting. FEMS Immunol Med Microbiol 1995; 10: 317–23.
14. Mohammadi M. Redline R, Nedrud J. Czinn S. Role of the host in pathogenesis of Helicobacter-associated gastritis: *H. felis* infection of inbred and coiigenic mouse strains. Infect Immun 1996; 64: 238–45.
15. Karita M. Kouchiyama T, Okita K, Nakazawa T. New small animal model for human gastric *Helicohacrer pylori* infection: success in both nude and euthymic mice. Am J Gastroenterol 1991: 86: 1596–603.
16. Kimura N, Ariga M, Icatlo FC Jr, et al. A euthymic hairless mouse model of *Helicohacter* ***pylori*** colonization and adherence to gastric epithelia1 cells in vivo. Clin Diagn Lab Immunol 1998; 5: 578–82.
17. Ferrero R, Thiberge JM, Huerre M, Labigne A. Immune responses of specific-pathogen-free mice to chronic *Helicobacter pylori* (strain SSI) infection. Infect Immun 1998; 66: 1349–55.
18. Michetti P. Wadstrom T, Kraehenbuhl JP. Lee A, Kreiss C, Blum AL. Frontiers in *Helicohacter pylori* research—pathogenesis; host response, vaccine development and new therapeutic approaches. Eur J Gastroenterol Hepatol 1996; 8: 717–22.
19. Lee A, O'Rourke J, Ungria MCD, Robertson B, Daskalopoulos G, Dixon MF. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology 1997: 112: 1386–97.
20. Soltez V, Schalen C, Mårdh PA. New selective medium for *Campylobacter pylori.* In: Kaijser B, Falsen E, eds. Proceedings of the Fourth International Workshop on Campylobacter infections. Gothenburg, Sweden: University of Gothenburg, 1988: 433–6.
21. Nilsson I. Ljungh A. Aleljung P, Wadstrom T. Immunoblot assay for serodiagnosis of *Helicohacter pylori* infections. J Clin Microbiol 1997: 35: 427–32.
22. Wang X, Sjunnesson H. Sturegård E, Wadstrom T, Willén R, Aleljung P. Dietary factors influence the recovery rates of *Helicohacter pylori* in a BALB/cA mouse model. Zent bl Bakteriol 1998; 288: 195–205.
23. Vandamme P, Glupczynski Y, Lage AP, Lammens C. Quint WG, Goossens H. Evaluation of random and repetitive motif primed polymerase chain reaction typing of *Helicobocter pylori.* Syst Appl Microbiol 1995; 18: 357–62.
24. Sturegård E, Sjunnesson H. Ho B, et al. Severe gastritis in guinea-pigs infected with *Helicobacter pylori.* J Med Microbiol 1998; 47: 1123–9.
25. Sakagami T, Dixon M, O'Rourke J, et al. Atrophic gastric changes in both *Helicohacter felis* and *Helicobater pylori* infected mice are host dependent and separate from antral gastritis. Gut 1996; 39: 639–48.
26. Berg DE, Hoffman PS, Appelmelk BJ, Kusters JG. The *Helicobacter pylori* genome sequence: genetic factors for long life in the gastric mucosa. Trends Microbiol 1997; 5: 468–74.

APMIS 108: 503–8, 2000
Printed in Denmark . All rights reserved

APMIS
ISSN 0903-4641

IV

Development of high-grade lymphoma in *Helicobacter pylori*-infected C57BL/6 mice

XIN WANG,[1] ROGER WILLÉN,[2] CHRISTINA ANDERSSON[3] and TORKEL WADSTRÖM[1]

[1]Department of Infectious Diseases & Medical Microbiology, [3]Department of Pathology, University of Lund, Lund and [2]Department of Pathology, Sahlgrenska University Hospital, Gothenburg, Sweden

Wang X, Willén R, Andersson C & Wadström T. Development of high-grade lymphoma in *Helicobacter pylori*-infected C57BL/6 mice. APMIS 2000;108:503–8.

Helicobacter pylori infection is associated with chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and MALT lymphoma. Mice with *H. pylori* infection develop severe gastritis and atrophic changes in their stomachs after 6 months. We followed *H. pylori*-infected animals for 13 months to find out whether dysplasia, carcinoma or lymphoma developed. Six-week-old C57BL/6 mice were infected with the CagA-positive and VacA-positive *H. pylori* mouse-passaged strain 119/95, fed a low antioxidant diet, and kept in microisolated cages. Histopathological changes were examined after 13 months' infection. All *H. pylori*-inoculated mice (n=5) developed a gastric squamous papilloma with nagging of the lamina muscularis after 13 months. Three out of five animals developed high-grade B-cell lymphoma derived from a MALT lymphoma at the squamous-corpus border with manifestations also in the liver, spleen and kidney. There was a suspicion of local gastric lymphoma in the two remaining mice but with no significant changes in the liver, spleen or kidney. The normal control mice showed no pathological changes in any of these organs. It is concluded that this mouse model with infection by the CagA-positive, vac-toxin-producing *H. pylori* strain 119/95 is suitable for use in the study of lymphoma development and also development of squamous cell papilloma with proliferative features.

Key words: Lymphoma; papilloma; *Helicobacter pylori*; C57BL/6 mice.

Torkel Wadström, Department of Infectious Diseases and Medical Microbiology, University of Lund, Sölvegatan 23, S-223 62 Lund, Sweden. e-mail: Torkel.Wadstrom@mmb.lu.se

Helicobacter pylori infection in man is associated with chronic type B gastritis, peptic ulcer disease, MALT (mucosa-associated lymphoid tissue) lymphoma and gastric carcinoma (1, 2). The histopathological features of low-grade primary gastric lymphoma resemble structures of Peyer's patches (1, 3–5). Transformation of low-grade MALT lymphoma to high-grade primary gastric lymphoma is well recognized and often a mixture of both can be found (4, 6, 7). Sometimes only a high-grade primary gastric lymphoma is found and therefore a de novo lymphoma cannot be ruled out (3). The MALT-lymphoma formation and growth is probably antigen driven and it has been suggested that *H. pylori* could serve as such. Neoplastic B cells are not immunoresponsive to *H. pylori* but their proliferation is dependent on cognate help from *H. pylori*-specific T-cells (8–10). B- and T-cells are recruited to the gastric mucosa as part of the complex immune response to *H. pylori*. Conversion to high-grade lymphoma might not require the presence of *H. pylori* (1, 3, 11).

We have previously shown that mice with *H. pylori* infection for 6 months develop severe gastritis and atrophic changes (12). In this study we followed *H. pylori* infection for 13 months in

Received December 22, 1999.
Accepted May 2, 2000.

C57BL/6 mice to elucidate whether malignancy might develop and thus provide us with a suitable model for pathogenesis studies of *H. pylori*-related lymphoma development and treatment studies.

MATERIALS AND METHODS

Bacterial strains

The *H. pylori* mouse-passaged strain 119/95 (a CagA+, VacA+ strain originally isolated from a duodenal ulcer patient at the University Hospital of Lund, Sweden) (13) was grown on GAB-Camp agar supplemented with 10% horse serum and incubated for 48 h at 37°C under microaerophilic conditions (14). The cells were harvested in PBS, centrifuged at 3000 rpm for 10 min, and resuspended in PBS to a final concentration of 10^9 colony-forming units (cfu)/ml.

Animals

Six- to eight-week-old conventional C57BL/6 mice were kept in microisolated cages with a 12-h light-dark cycle and provided with a rat and mouse standard diet no. 2 expanded (B&K Universal Company, Stockholm, Sweden) (15) and water ad libitum. Mice were inoculated with 0.3 ml of *H. pylori* suspension three times at 2-day intervals. After 13 months, mice (n=2 normal control group and n=5 *H. pylori*-inoculated group) were killed using carbon dioxide and blood was drawn by heart puncture. Stomach, jejunum and ileum, colon, liver and kidney were collected for histopathology.

Histopathology and immunohistochemistry

Murine stomach, liver, spleen and kidney tissues were fixed in 10% buffered formalin, embedded in paraffin, and 4 μm sections were prepared, stained with hematoxylin-eosin using standard procedures. A new classification of gastric lymphoma has been published (5, 16) and grading of the lymphoma (grades 0–5) was according to Wotherspoon et al. (16).

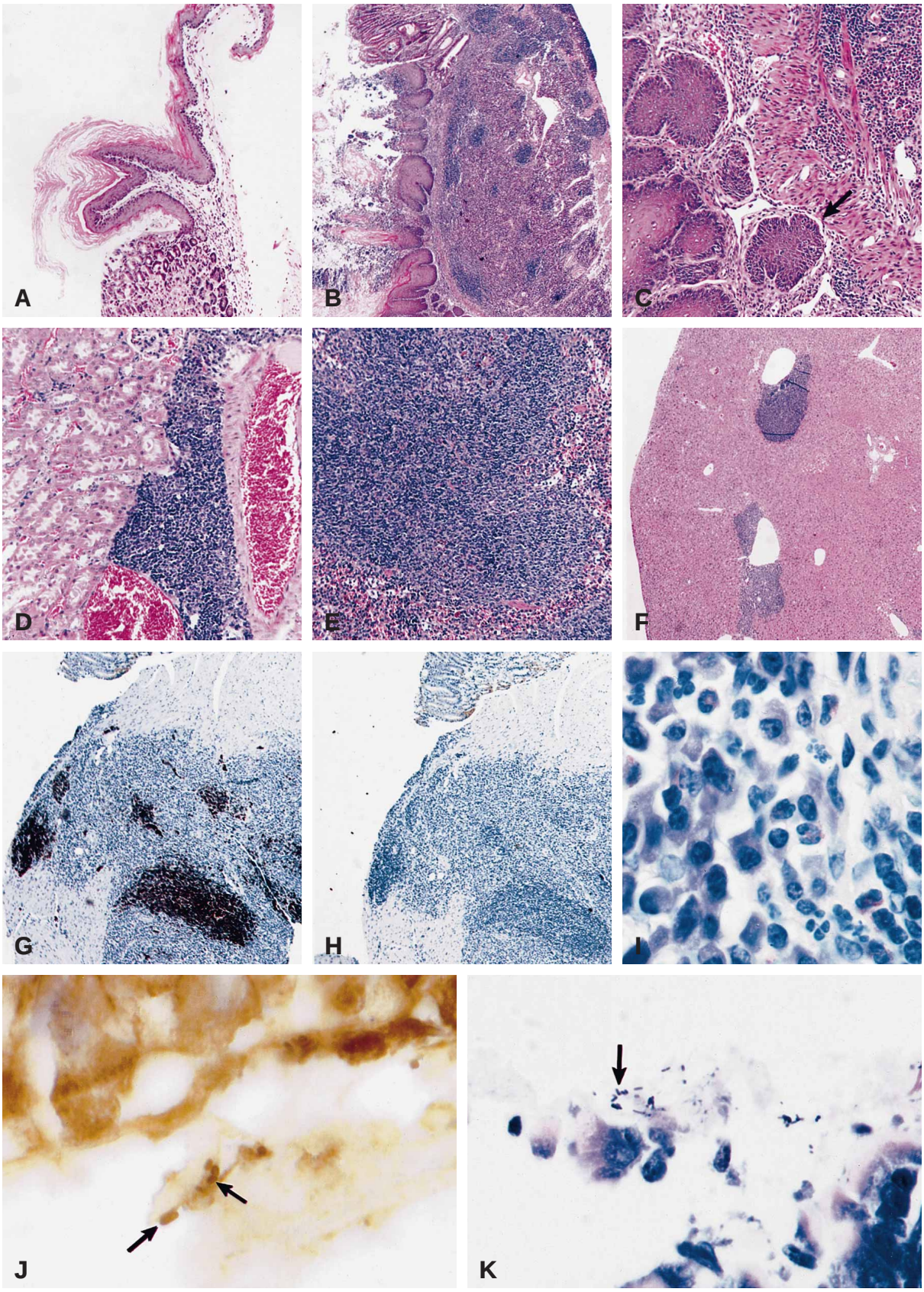
Lymphoma tissues were subjected to immunohistopathology with markers for T-cells (CD3) (Cedarlane, dilution 1:20) in an avidin-biotin system as well as antibody against B-cell tissue CD45R (Cedarlane, dilution 1:300). To visualize the presence of intraepithelial lymphocytes the epithelial marker AE1/AE3 (DAKO, dilution 1:100) was used. To show the presence of *H. pylori*, hematoxylin-eosin, Giemsa and immunostaining (anti-Hp, DAKO, dilution 1:20) were used. The density was evaluated according to Dixon et al. (17)

RESULTS

The normal control animal demonstrated a well-developed corpus mucosa with specialized cells and normal foveolar surface (Fig. 1A). The canalis was normal, covered with foveolar cells, and no inflammatory cell reaction was noticed. In the proximal part of the gastric tissue the mucosa was covered by squamous epithelium. In the inflow area of the esophagus into the gastric area, papillomatous-like tissue was seen covered by squamous epithelium. In that area normally a slight increase in lymphoplasmocytic cells was noted in the stroma. Slides from liver, spleen and kidney showed a normal picture without any lymphocytic or abnormal cell infiltration.

Animals infected by *H. pylori* for 13 months demonstrated a completely different feature (Fig. 1 B & C). In the proximal gastric area with a squamous cell surface a profound inflammatory cell reaction was found. The infiltration consisted of highly abnormal lymphocytes and these cells demonstrated rough chromatin, rich mitosis, and uneven nuclear and cell borders; often several nucleoli were noted (Fig. 1I). Lymphoepithelial lesions (LEL) were seen in both the squamous cell populations as well as in the surface and lower part of the gastric mucosa. The lymphoma seems to be derived from MALT-omas with subsequent dedifferentiated lymphomatous parts. It grew diffusely in the squamous covered mucosa, the corpus mucosa, and showed deep infiltration into the ventricular wall. The tumor was also seen growing outside the gastric tissue partly surrounding the esophagus-gastric junction. Local lymph glands were most probably also involved.

Fig. 1. A: Normal mouse gastric mucosa with corpus mucosa and squamous upper part (×5); B: Lymphoma infiltrate in mucosa, submucosa and muscular tissue (×2.5); C: Epithelial proliferation with nagging of lamina muscularis mucosa (×5); D: Lymphoma infiltrate in kidney tissue (×10); E: Lymphoma infiltrate in spleen (×10); F: Lymphoma infiltrate in liver tissue (×10); G: CD45R immunostain for B-lymphocytes positive (×5); H: CD3 immunostain for T-lymphocytes negative (×5); I: Higher magnification of lymphoma cell infiltrate. Mixture of normal lymphocytes and plasmacytoid blastic cells (×100); J: Immunostain for *H. pylori*. Curved bacteria at arrows (×100); K: Giemsa stain for *H. pylori* (arrow) (×60).


A
B
C
D
E
F
G
H
I
J
K

In the squamous area a squamous papillomatous proliferation was seen with hyper- and parakeratosis, acanthosis and downbulging squamous epithelium. From time to time seemingly free-floating squamous cell areas were noted. These squamous cell areas were seen bulging into the lamina muscularis mucosa but not penetrating it. The cell tissue was mostly indolent and sometimes mitosis was noted. It was thus not clear if we had a squamous papilloma with a tendency to proliferation or a very highly differentiated squamous carcinoma. Infiltrates of lymphoblastic cells were also noted in kidney, spleen and liver (Fig. 1D, E & F). The lymphoma tissue was subjected to immunohistopathology with markers for T-cells (CD3) (Fig. 1H) as well as antibody against B-cell tissue CD45R (Fig. 1G). The reactivity for T-cells was basically negative while reactivity for B-cells was strongly positive. AE1/AE3 for intermediate epithelial filaments helped to demonstrate the presence of LEL. LEL were found in both the squamous as well as in the glandular cells.

Hematoxylin-eosin stain and Giemsa stain for gastric tissue as well as antibodies for *H. pylori* showed a few *H. pylori* in the mucus as well as in some glands (Fig. 1, J & K).

Grading of the lymphomas according to Wotherspoon et al. (16) revealed three cases of grade 5 and two cases of grade 4. No lymphoma (grade 0) was noted in the control group.

DISCUSSION

C57BL/6 mice subjected to *H. pylori* infection for one week and then kept for another 13 months as described previously (13) developed two main changes: squamous papilloma-like structures with growth pattern to some extent mimicking highly differentiated squamous cell carcinoma and blast-like high-grade lymphoma positive for B-cell marker showing highly abnormal cells and mitosis. The tumor cell population destroyed the local mucosa and muscular tissue and was seen growing outside the gastric tissue. Moreover, heavy infiltration of the same cell population in liver, spleen and kidney tissue clearly demonstrated the malignant features of the lymphoma.

H. pylori usually creates a low-grade lymphoma, the so-called MALT lymphoma, in human as well as animal models (8, 18, 19). There are also reports of high-grade lymphoma as well as a mixture of high- and low-grade lymphoma in both human and mouse systems (1, 11, 18). De novo high-grade lymphoma sometimes occurs (1). It is concluded that our mouse model exposed to *H. pylori* strain 119/95, a CagA-positive and vac toxin-producing strain, is another good example of an animal model suitable for studying lymphoma development.

Squamous cell proliferation can also be stimulated in this model at least up to a squamous cell papilloma with proliferative features. In this study we were not able to find manifest infiltrative carcinoma (20), but the pushing borders clearly demonstrated the proliferative capacity of the tumors. It might be argued that an agent other than *H. pylori* in the present setting might be the cause of lymphoma and squamous cell proliferation. However, parallel to this study we had several groups of mice – controls and other long-term food treatment groups – at the same facility during the same time period. None of these animals in the different treatment groups developed a lymphoma or any squamous cell proliferation. It is therefore unlikely that the *H. pylori* does not play any part in the tumor development. There are few animals in this preliminary study due to the fact that these results appeared in one of many groups of animals in a study of different antioxidant treatment in combination with *H. pylori*. This reduces the possibility of a more certain interpretation. Further studies with more animals to determine the value of this animal model are, however, ongoing in our laboratory.

H. pylori can be identified in more than 90% of cases of gastric MALT lymphoma (6, 16). In more advanced cases fewer *H. pylori* are found (21). This suggests that *H. pylori* is more closely associated with the precursor or initial genesis of MALT lymphoma (2). High-grade MALT lymphoma transformation may be more likely to occur following infection with CagA+ strains of *H. pylori* (22). The strain used in this study was both CagA and VacA positive. Grading according to Wotherspoon et al. (16) revealed definite or suspicion of local gastric lymphoma with a B-cell pattern. However, in humans few cases of T-cell lymphoma have been

reported (18, 23). We did not stain for *kappa* or *lambda* chains as monoclonality can also be seen in benign gastric lymphoid infiltration (24). Moreover, more than 95% of immunoglobulins contain *kappa* chains in the normal mouse (19), so it would be difficult to detect minor deviations from that with the present immune technique (2). The mechanisms behind the decrease in *H. pylori* during lymphoma formation in man are uncertain. Mucosal atrophy, intestinal metaplasia and reduction of neutrophils have been suggested. The fact that antibiotic treatment can be successful in the earlier phase of low-grade MALT lymphoma (16), while high-grade and more advanced lymphoma with few *H. pylori* are usually non-responders, may be attributed to a sequence of molecular events (1, 4, 25, 26). Immunological drive with *H. pylori*-specific T cells is diminished with lack of *H. pylori* bacteria (11). More advanced molecular events beyond trisomy 3, translocation 1;14 and changes of *p*53 mutant detection, seem to be a process no longer *H. pylori* dependent (1, 21, 26). Thus, no further clinical effect of antibiotics is to be expected.

In our study using hematoxylin-eosin, Giemsa and specific *H. pylori* immunostaining no bacteria were found in the control group while in the treatment group a few *H. pylori*-like bacteria were seen.

In order to further evaluate the molecular events, genetics and treatment regimen attempts it is of value to have a model system which is easy to handle. Lymphoma development occurred over a shorter time period in this model than in earlier published studies (11, 19).

Spontaneous lymphomas are known to appear in aged mice (27): average incidence after 20 months in Balb/c mice is 16%. Enno et al. (19) reported equal numbers of malignant lymphoma in test and control groups in the spleen, but gastric MALT-omas were only observed in *Helicobacter*-infected animals. In an extended study (11) gastric lymphomas were also seen, but these were not of a MALT type. Our animals were not old and other groups of animals kept for the same period of time did not demonstrate lymphomatous changes. Only the *H. pylori*-treated group showed lymphomatous changes, which speaks in favor of an *H. pylori*-related cause of lymphoma development.

H. pylori can be found in combination with gastric carcinoma (25). Coexisting adenocarcinoma and lymphoma have been reported; in this study we found high-grade B-cell lymphoma derived from MALT lesions together with highly proliferative squamous cells with a papillomatous structure and expanding borders, though with no definite carcinoma. Whether this model will also be suitable for studying the carcinoma development remains to be elucidated.

This work was supported by grants from the Swedish Medical Research Council (16X04723), Swedish Forestry Agriculture Research Council (50.0497/98), Kungliga fysiografiska sällskapet in Lund, John and Augusta Perssons Foundation in Lund, the Medical Faculty, Lund University, and AstaCarotene AB, Gustavsberg, Sweden,
We would like to thank Ulric Pedersen for the skilled photography.

REFERENCES

1. Isaacson PG. Recent developments in our understanding of gastric lymphomas. Am J Surg Pathol 1996;20:S1–7.
2. Nakamura S, Aoyagi K, Furuse M, Suekane H, Matsumoto T, Yao T, et al. B-cell monoclonality precedes the development of gastric MALT lymphoma in *Helicobacter pylori*-associated chronic gastritis. Am J Pathol 1998;152:1271–9.
3. Bouzourene H, Haefliger T, Delacretaz F, Saraga E. The role of *Helicobacter pylori* in primary gastric MALT lymphoma. Histopathology 1999;34: 118–23.
4. De Wolf-Peeters C, Achten R. The histogenesis of large-cell gastric lymphomas. Histopathology 1999;34:71–5.
5. Hsi ED, Eisbruch A, Greenson JK, Singleton TP, Ross CW, Schnitzer B. Classification of primary gastric lymphomas according to histologic features. Am J Surg Pathol 1998;22:17–27.
6. Chan JK, Ng CS, Isaacson PG. Relationship between high-grade lymphoma and low-grade B-cell mucosa-associated lymphoid tissue lymphoma (MALToma) of the stomach. Am J Pathol 1990;136:1153–64.
7. Matolcsy A, Nagy M, Kisfaludy N, Kelenyi G. Distinct clonal origin of low-grade MALT-type and high-grade lesions of a multifocal gastric lymphoma. Histopathology 1999;34:6–8.
8. Hussell T, Isaacson PG, Crabtree JE, Spencer J. The response of cells from low-grade B-cell gastric lymphomas of mucosa-associated lymphoid tissue to *Helicobacter pylori*. Lancet 1993,342: 571–4.
9. Hussell T, Isaacson PG, Crabtree JE, Dogan A,

Spencer J. Immunoglobulin specificity of low grade B cell gastrointestinal lymphoma of mucosa-associated lymphoid tissue (MALT) type. Am J Pathol 1993;142:285–92.
10. Hussell T, Isaacson PG, Crabtree JE, Spencer J. *Helicobacter pylori*-specific tumour-infiltrating T cells provide contact dependent help for the growth of malignant B cells in low-grade gastric lymphoma of mucosa-associated lymphoid tissue. J Pathol 1996;178:122–7.
11. Enno A, O'Rourke J, Braye S, Howlett R, Lee A. Antigen-dependent progression of mucosa-associated lymphoid tissue (MALT)-type lymphoma in the stomach. Effects of antimicrobial therapy on gastric MALT lymphoma in mice. Am J Pathol 1998;152:1625–32.
12. Wang X, Willén R, Wadström T. Gastric dysplasia due to *H. pylori* infection and carcinogenic substances in mice. Gut (Suppl) 1998;43:A69.
13. Wang X, Willén R, Wadström T, Aleljung P. RAPD-PCR, histopathological and serological analyis of four mouse strains infected with multiple strains of *Helicobacter pylori*. Microbial Ecol Health Dis 1998;10:148–54.
14. Wang X, Sturegard E, Rupar R, Nilsson HO, Aleljung PA, Carlén B, et al. Infection of BALB/c A mice by spiral and coccoid forms of *Helicobacter pylori*. J Med Microbiol 1997;46:657–63.
15. Wang X, Sjunnesson H, Sturegard E, Wadström T, Willén R, Aleljung P. Dietary factors influence the recovery rates of *Helicobacter pylori* in a BALB/c A mouce model. Zentralbl Bakteriol 1998;288:195–205.
16. Wotherspoon AC, Doglioni C, Diss TC, Pan L, Moschini A, de Boni M, et al. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. Lancet 1993;342:575–7.
17. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol 1996;20:1161–81.
18. Isaacson PG. Gastrointestinal lymphoma. Hum Pathol 1994;25.1020–9.
19. Enno A, O'Rourke JL, Howlett CR, Jack A, Dixon MF, Lee A. MALToma-like lesions in the murine gastric mucosa after long-term infection with helicobacter felis. A mouse model of *Helicobacter pylori*-induced gastric lymphoma. Am J Pathol 1995;147:217–22.
20. Rokkas T, Wotherspoon A. Carcinogenesis. Curr Opin Gastroenterol 1999;15(Suppl 1):S23–8.
21. Dayal Y. Introduction. Am J Surg Pathol 1996;20(Suppl 1):iv–vi.
22. Peng H, Ranaldi R, Diss TC, Isaacson PG, Bearzi I, Pan L. High frequency of CagA+ *Helicobacter pylori* infection in high-grade gastric MALT B-cell lymphomas. J Pathol 1998;185:409–12.
23. Foss HD, Schmitt-Graff A, Daum S, Anagnostopoulos I, Assaf C, Hummel M, et al. Origin of primary gastric T-cell lymphomas from intraepithelial T-lymphocytes report of two cases. Histopathology 1999;34:9–15.
24. Savio A, Franzin G, Wotherspoon AC, Zamboni G, Negrini R, Buffoli F, et al. Diagnosis and posttreatment follow-up of *Helicobacter pylori*-positive gastric lymphoma of mucosa-associated lymphoid tissue: histology, polymerase chain reaction, or both? Blood 1996;87:1255–60.
25. Eidt S, Stolte M. The significance of *Helicobacter pylori* in relation to gastric cancer and lymphoma. Eur J Gastroenterol Hepatol 1995;7:318–21.
26. Du M, Peng H, Singh N, Isaacson PG, Pan L. The accumulation of *p*53 abnormalities is associated with progression of mucosa-associated lymphoid tissue lymphoma. Blood 1995;86:4587–92.
27. Dunn TB, Deringer MK. Reticulum cell neoplasm, type B, or the "Hodgkin's-like lesion" of the mouse. J Natl Cancer Inst 1968;40:771–821.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2000, p. 2452–2457 Vol. 44, No. 9
0066-4804/00/$04.00+0

V

Astaxanthin-Rich Algal Meal and Vitamin C Inhibit *Helicobacter pylori* Infection in BALB/cA Mice

XIN WANG,[1] ROGER WILLÉN,[2] AND TORKEL WADSTRÖM[1]*

Department of Infectious Diseases and Medical Microbiology, University of Lund, Lund,[1] and Department of Pathology, Sahlgrenska University Hospital, Gothenburg,[2] Sweden

Received 28 December 1999/Returned for modification 10 April 2000/Accepted 19 June 2000

***Helicobacter pylori* infection in humans is associated with chronic type B gastritis, peptic ulcer disease, and gastric carcinoma. A high intake of carotenoids and vitamin C has been proposed to prevent development of gastric malignancies. The aim of this study was to explore if the microalga *Haematococcus pluvialis* rich in the carotenoid astaxanthin and vitamin C can inhibit experimental *H. pylori* infection in a BALB/cA mouse model. Six-week-old BALB/cA mice were infected with the mouse-passaged *H. pylori* strain 119/95. At 2 weeks postinoculation mice were treated orally once daily for 10 days (i) with different doses of algal meal rich in astaxanthin (0.4, 2, and 4 g/kg of body weight, with the astaxanthin content at 10, 50, and 100 mg/kg, respectively), (ii) with a control meal (algal meal without astaxanthin, 4 g/kg), or (iii) with vitamin C (400 mg/kg). Five mice from each group were sacrificed 1 day after the cessation of treatment, and the other five animals were sacrificed 10 days after the cessation of treatment. Culture of *H. pylori* and determination of the inflammation score of the gastric mucosae were used to determine the outcome of the treatment. Mice treated with astaxanthin-rich algal meal or vitamin C showed significantly lower colonization levels and lower inflammation scores than those of untreated or control-meal-treated animals at 1 day and 10 days after the cessation of treatment. Lipid peroxidation was significantly decreased in mice treated with the astaxanthin-rich algal meal and vitamin C compared with that of animals not treated or treated with the control meal. Both astaxanthin-rich algal meal and vitamin C showed an inhibitory effect on *H. pylori* growth in vitro. In conclusion, antioxidants may be a new strategy for treating *H. pylori* infection in humans.**

The human gastric pathogen *Helicobacter pylori* causes type B chronic gastritis and is associated with peptic ulcer disease as well as gastric cancer (7, 9, 38). Some epidemiological studies have shown the difference between the prevalence of *H. pylori*-associated diseases and intake of certain vitamins and antioxidants in various populations (10, 28, 33) and that a high intake of vitamin C, α-tocopherol, or β-carotene in food reduces the risk of gastric carcinoma (2, 30). *H. pylori* infection in humans is characterized by a marked infiltration of neutrophilic leukocytes of the gastric mucosa, and the generation of reactive oxygen metabolites (ROMs) may play a part in the development of severe chronic type B gastritis (15, 26).

Astaxanthin is a carotenoid found in many different organisms in nature, but the main dietary sources for humans are found in crustaceans and other seafood, especially salmon. Astaxanthin is a powerful lipid-soluble antioxidant in vitro (8, 16, 19) and was shown to be most effective in stimulating immune defenses when different carotenoids were compared (12, 13, 23). Vitamin C is a water-soluble antioxidant required for many biological functions such as the normal synthesis of hormones, neurotransmitters, collagen, and carnitine and the absorption of iron and other substances (17, 27). Vitamin C is a dietary antioxidant which, at a normal physiologic concentration, scavenges ROMs to provide protection against oxidative DNA damage (6, 21). The aim of this study was to explore how the antioxidant astaxanthin from the alga *Haematococcus pluvialis* and vitamin C affect *H. pylori* infection in a BALB/cA mouse model.

* Corresponding author. Mailing address: Department of Infectious Diseases and Medical Microbiology, University of Lund, Sölvegatan 23, S-223 62 Lund, Sweden. Phone: 46 46 17 32 40. Fax: 46 46 15 25 64. E-mail: Torkel.Wadstrom@mmb.lu.se.

MATERIALS AND METHODS

Chemicals. Homogenized and dried cells of the unicellular green alga *Haematococcus pluvialis* rich in astaxanthin (2 to 3% [wt/wt]) and algal meal without astaxanthin as the control meal were obtained from AstaCarotene AB (Gustavsberg, Sweden). Vitamin C (L-ascorbic acid) was purchased from ICN Biomedicals Inc. (Lund, Sweden). Algal meal and control meal were suspended in distilled water and vitamin C was dissolved in distilled water just before use.

Bacterial strains. The *H. pylori* mouse-passaged strain 119/95 was grown on GAB-Camp agar (Gc Agar Base; Becton Dickinson, Lund, Sweden) supplemented with 10% horse serum and incubated for 48 h at 37°C under microaerophilic conditions (35). The cells were harvested in phosphate-buffered saline (PBS), centrifuged at 2,800 × *g* for 10 min, and resuspended in PBS to a final concentration of 10^9 CFU/ml.

Animals. Six- to eight-week-old conventional BALB/cA mice were used in this study (B&K Universal Company, Stockholm, Sweden). Mice were housed on a 12-h light–12-h dark schedule and fed with rat and mouse standard diet no. 2 (B&K Universal) (34) and water ad libitum.

Experimental design. Sixty mice were inoculated orally through a feeding tube three times at 2-day intervals with 0.1 ml of an *H. pylori* suspension containing 10^9 CFU/ml, and 10 mice were inoculated with PBS as a negative control group. The *H. pylori*-inoculated mice were divided into six groups. Five groups were orally treated with three doses of algal meal rich in astaxanthin (0.4, 2, and 4 g/kg of body weight, in which the astaxanthin content was 10, 50, and 100 mg/kg, respectively), a control algal meal (4 g/kg), or vitamin C (400 mg/kg) once daily for 10 days at 2 weeks postinoculation (p.i.). The infected and normal uninfected control mice were given distilled water through a feeding tube. Half of the animals from each group were sacrificed 1 day after the cessation of treatment, and the other half were sacrificed 10 days after the cessation of treatment.

Mice were killed with carbon dioxide, and their stomachs were collected. The stomachs were opened through the longer curvature using sterile surgical instruments. One-third of the stomachs and duodena, covering all subtypes of mucosa, were used for histopathology. One-third of the stomachs were used for culturing *H. pylori*. The remaining part of the gastric tissue was used for determination of the astaxanthin concentration.

Culture. One-third of stomach biopsies were placed in 0.5 ml of PBS in a 1.5-ml Eppendorf tube and homogenized with a Pellet Pestle Mixer from KEBO Laboratories (Lund, Sweden). The homogenized sample was serially diluted 10-fold. Each 0.1 ml of homogenate was plated on GAB-Camp agar and incubated at 37°C for 5 to 10 days under microaerophilic conditions (36). The *H. pylori* colonies were counted and calculated as $\log_{10}$ CFU per milliliter of homogenate. The presence of *H. pylori* on the culture plates was confirmed by

TABLE 1. Concentrations of total carotenoids and astaxanthin in mouse stomachs

Treatment group (concn of astaxanthin [mg/kg])	Mean concn ± SD (μg/kg)			
	1 day after cessation of treatment		10 days after cessation of treatment	
	Total carotenoid	Astaxanthin	Total carotenoid	Astaxanthin
Normal	58 ± 8	0	51 ± 3	0
H. pylori	98 ± 28	0	58 ± 5	0
H. pylori + algal meal (100)	5,806 ± 1,162	516 ± 192	164 ± 34	49 ± 10[a]
H. pylori + algal meal (50)	5,225 ± 1,344	344 ± 101	107 ± 13	26 ± 3
H. pylori + algal meal (10)	2,926 ± 668	120 ± 29[b]	116 ± 34	34 ± 15

[a] $P < 0.05$ versus results from the group treated with 50 mg of astaxanthin per kg.

[b] $P < 0.05$ versus results from the groups treated with 100 and 50 mg of astaxanthin per kg.

urease, catalase, and oxidase testing, Gram staining, and PCR analysis with *H. pylori* urease primers (20).

Carotenoid and astaxanthin analysis. Stomach samples were homogenized and extracted in acetone (25). The acetone extracts were pooled and mixed vigorously with cyclohexane (1:1) and approximately 200 to 400 μl of distilled water to obtain phase separation. The samples were then centrifuged, and the concentrations of carotenoids recovered in the hexane phase were determined by measuring the absorbency at 474 nm with a Spectronic 601 spectrophotometer (Milton Roy Co.). An extinction coefficient measured in micrograms per kilogram was used for calculations.

The carotenoid composition was determined by high-pressure liquid chromatography after evaporation of the cyclohexane extract to dryness with nitrogen and dissolving of the carotenoids in chloroform-methanol (2:1). The high-pressure liquid chromatography system (Merck-Hitachi) consisted of a model L6200A Intelligent pump, a model D-2000 injector, and a model L4200 visible light-UV detector set at a wavelength of 474 nm and with 0.1 absorbency unit at full scale. External and internal carotenoid standards (astaxanthin and canthaxanthin, 99% pure; Hoffman-La Roche Ltd., Hvidovre, Denmark) were employed to check the recovery of carotenoid during extraction and the reproducibility of the results of the analytical methods applied. All the solvents and chemicals used were of analytical grade and purchased from Merck, Darmstadt, Germany.

Histopathology. Murine stomach tissues were fixed in 10% buffered formalin and embedded in paraffin, and 4-μm-thick sections were prepared and stained with hematoxylin and eosin by standard procedures. The degree of inflammation was scored in a blind manner on a scale of 0 to 3 for body, antrum, and duodenum (36).

Lipid peroxidation assay. Mice stomach tissues were homogenized in 20 mM Tris-HCl, pH 7.4, to a concentration of 10% (wt/vol). Homogenate supernatants (200 μl) were tested for malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal with a lipid peroxidation assay kit from Calbiochem (Lund, Sweden). The colorimeters were measured at an absorbency at 586 nm, and tissue lipid peroxidation was calculated as micromoles per gram of tissue.

In vitro inhibition test. MICs were determined as described elsewhere (22) such that each preculture containing 10^3 cells was plated onto GAB-Camp agar with or without various concentrations of algal meal (0.3125 to 20 mg/ml), with algal meal without astaxanthin (5 mg/ml), or with vitamin C (0.5 to 4 mg/ml). The surviving cells were counted on the agar as colonies, and the MIC was defined as the concentration leaving no survivors after 5 to 10 days of incubation under microaerophilic conditions. Ten strains of *H. pylori* were tested, and MICs were shown as a range.

Statistical analysis. The Mann-Whitney U test was used for analysis of colonization and inflammation distribution. The level of significance was chosen to be a P of <0.05.

RESULTS

Total carotenoid and astaxanthin analysis. The mouse stomachs showed correspondingly high total carotenoid and astaxanthin contents when they were treated with various concentrations of astaxanthin (Table 1). Significant differences were noted between the treated and untreated group, especially for animals just posttreatment. Mice treated with the highest dose of astaxanthin demonstrated a higher astaxanthin content in their stomachs than those of the animals treated with lower doses.

Culture. All noninoculated mice were *H. pylori* negative in culture. Both astaxanthin-rich algal meal (dose from 10 to 100 mg/kg) and vitamin C significantly reduced the number of *H. pylori* organisms in gastric tissue 1 day after the cessation of treatment (3.5 weeks p.i.) compared with the numbers recoverable from the untreated mice and the control mice treated with meal lacking astaxanthin ($P < 0.05$) (Fig. 1). At 10 days after the cessation of treatment (5 weeks p.i.), the numbers of *H. pylori* organisms in the groups treated with astaxanthin-rich algal meal and vitamin C were again significantly lower than the numbers in the groups not treated or treated with the control meal ($P < 0.05$) (Fig. 1). However, the astaxanthin-rich algal meal (100 mg/kg) and vitamin C (400 mg/kg) treatment groups had more numbers of *H. pylori*-negative animals (40%)

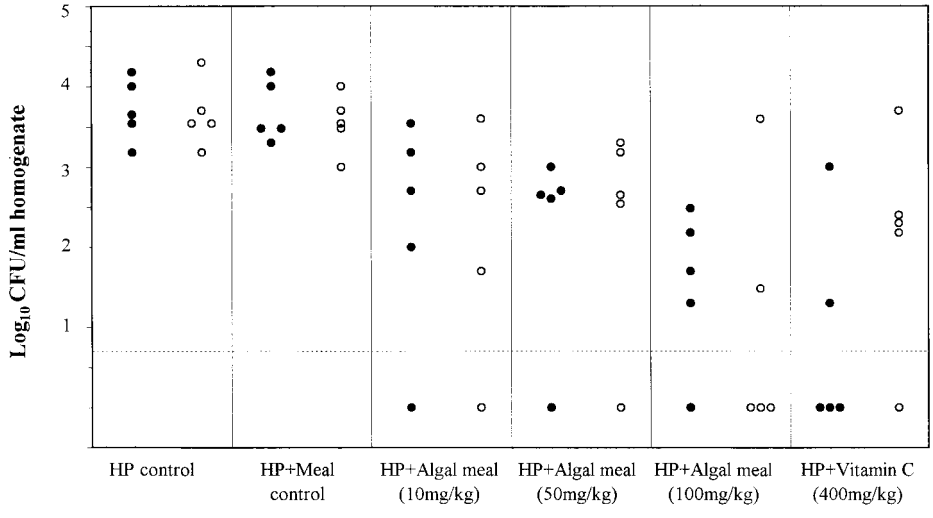


FIG. 1. Effects of different doses of astaxanthin-rich algal meal or vitamin C on the recovery of *H. pylori* from BALB/cA mice. Treatment was started 2 weeks p.i., and samples were taken 3.5 (●) and 5 (○) weeks p.i. Each dot or circle represents the bacterial count from one animal, and the dashed line indicates the limit of detection. HP, *H. pylori*.

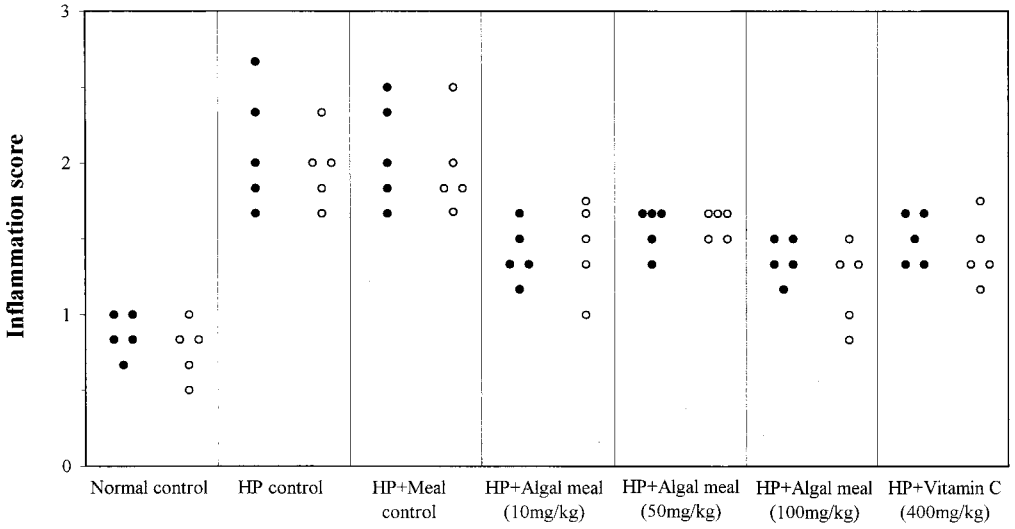


FIG. 2. Inflammation scores for gastritis in BALB/cA mice treated with astaxanthin-rich algal meal and vitamin C at 3.5 (●) and 5 (○) weeks p.i. Each dot or circle represents the inflammation score from one animal. HP, *H. pylori*.

than the astaxanthin-rich algal meal (10 and 50 mg/kg) treatment groups (20%). There were no significant differences among the groups treated with three doses of astaxanthin-rich algal meal and vitamin C.

Histopathology. Mice treated with astaxanthin-rich algal meal or vitamin C showed significantly lower inflammation scores than control mice infected with *H. pylori* or treated with meal lacking astaxanthin 1 day and 10 days after the cessation of treatment ($P < 0.05$) (Fig. 2). The control-meal-treated mice developed gastritis as severe as that of the untreated control animals, and their inflammation scores were significantly higher than those of the non-*H. pylori*-inoculated mice ($P < 0.01$). The mice treated with the highest dose of astaxanthin (100 mg/kg) in algal meal showed significantly lower inflammation scores than the mice treated with 50 mg of astaxanthin per kg ($P < 0.05$).

Normal noninfected control mice showed normal fundic mucosae (Fig. 3A). Mice treated with astaxanthin-rich algal meal (100 mg/kg) or vitamin C (400 mg/kg) showed fewer inflammatory cells in their mucosae than infected control mice (Fig. 3B to D).

Lipid peroxidation. The concentrations of MDA and 4-hydroxyalkenals in murine stomachs (in micromoles per gram of tissue) were significantly increased in *H. pylori*-infected untreated and control-meal (algal meal without astaxanthin)-treated mice compared with concentrations in normal control animals ($P < 0.01$). All astaxanthin-rich algal meal- or vitamin C-treated mice showed significant decreases in lipid peroxidation compared with levels in the untreated and control-meal-treated animals ($P < 0.05$) (Fig. 4).

In vitro inhibition. Astaxanthin-rich algal meal inhibited *H. pylori* growth at 0.3125 to 2.5 mg/ml (astaxanthin content, 6.25 to 50 μg/ml, pH 7.2), while algal meal without astaxanthin did not show this effect at 5 mg/ml. Vitamin C inhibited the growth of *H. pylori* at concentrations of 0.5 to 2 mg/ml (pH 7.2).

DISCUSSION

We have shown that antioxidants such as algal meal rich in astaxanthin as well as vitamin C inhibit *H. pylori* infection in BALB/cA mice. Among the three doses of astaxanthin tested, the highest dose (100 mg/kg) showed the best effect in reducing bacterial load and gastric inflammation. This finding is to our knowledge the first demonstration of an antimicrobial activity of astaxanthin-rich algal meal against *H. pylori* and associated gastric inflammation.

H. pylori infection has been associated with a decreased level of vitamin C and of major antioxidants (e.g., β-carotene) in human gastric tissue (5, 26). We found that vitamin C reduced bacterial colonization in the murine stomach and decreased the inflammation score. Interestingly, Jarosz et al. (11) reported that a high daily dose of vitamin C for 4 weeks (5 g per day) given to *H. pylori*-infected patients with chronic gastritis resulted in apparent *H. pylori* eradication in 30% of treated patients. In those patients the highly significant rise in total vitamin C concentration in the gastric juice persisted for at least 4 weeks posttreatment. Vitamin C not only seems to be an antioxidant and a free radical scavenger (17, 21, 26) but also shows antimicrobial activity against *H. pylori* both in vitro and in a Mongolian gerbil infection model (39).

Epidemiological evidence and clinical experiments suggest that vitamin C may exert a protective effect against the development of *H. pylori*-associated gastric carcinoma (4, 6, 37), but the mechanisms involved are not so clear.

The carotenoid astaxanthin has been established to be a powerful antioxidant in vitro (15, 24) and was previously shown to be able to prevent oral carcinogenesis in an experimental rat model (32). However, this carotenoid has not previously been shown to have an antimicrobial activity. We found that algal meal rich in astaxanthin has an inhibitory effect on *H. pylori* growth in vitro and also colonization in mouse stomach. BALB/cA mice treated with astaxanthin-rich algal meal showed decreased lipid peroxidation and granulocyte infiltration in their gastric mucosae.

H. pylori-infected individuals show high oxidative stress and high levels of ROMs in their gastric mucosae and an increased gastric antioxidative capacity after the eradication of *H. pylori* (14). A recent study of the formation of pro- and antioxidants to *H. pylori* infection in a Mongolian gerbil model showed an increase in the level of lipid peroxidation and activated glutathione turnover (31).

Astaxanthin acts as an antioxidant that protects against tis-

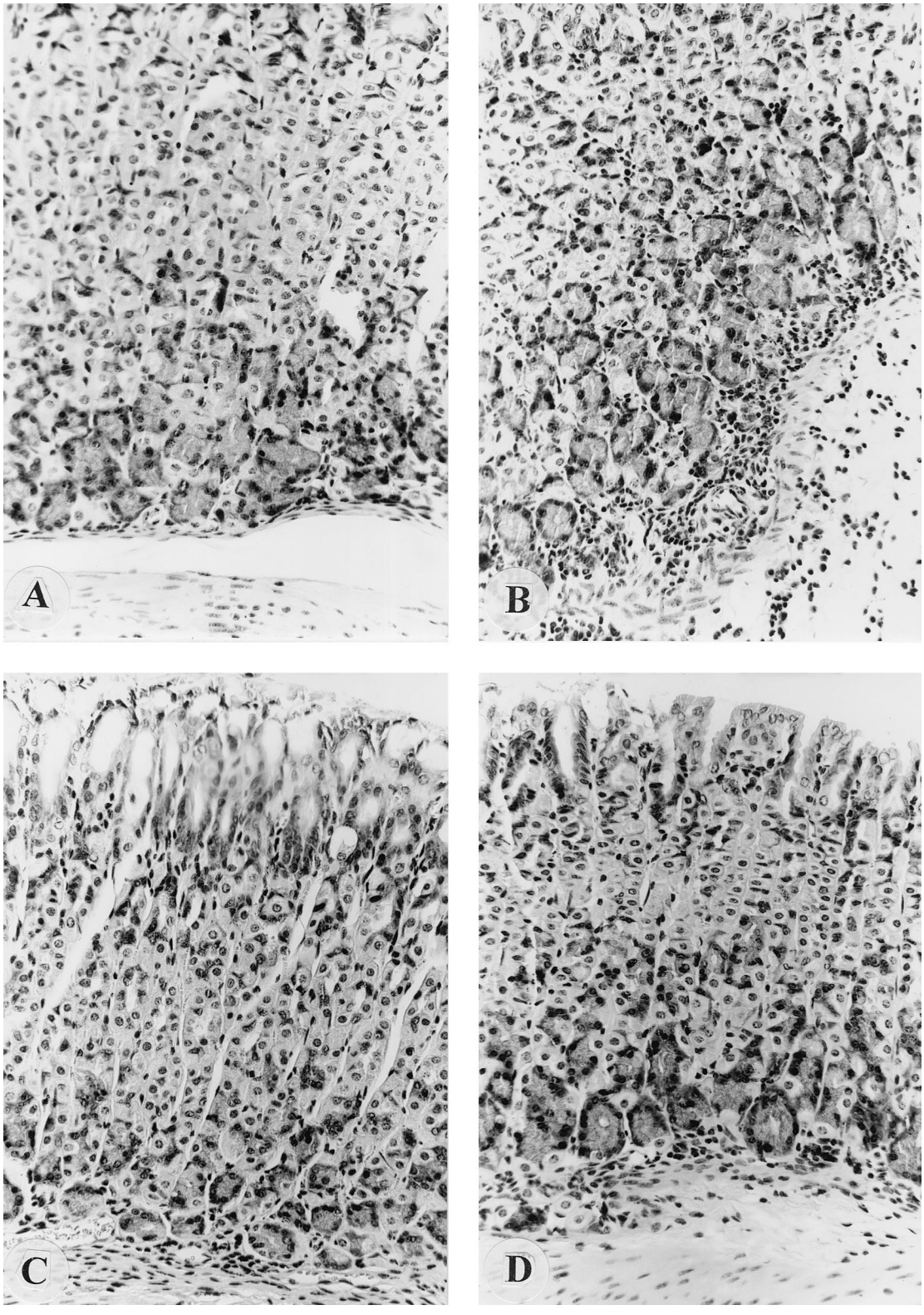


FIG. 3. (A) Normal fundic mucosa from an uninfected control mouse; (B) tissue from an *H. pylori*-infected mouse with a large amount of acute inflammatory cell infiltration within the mucosa and along the lamina muscular mucosa; (C and D) less inflammation (small amount of inflammatory cell infiltration) in mice treated with astaxanthin-rich algal meal and vitamin C, respectively.

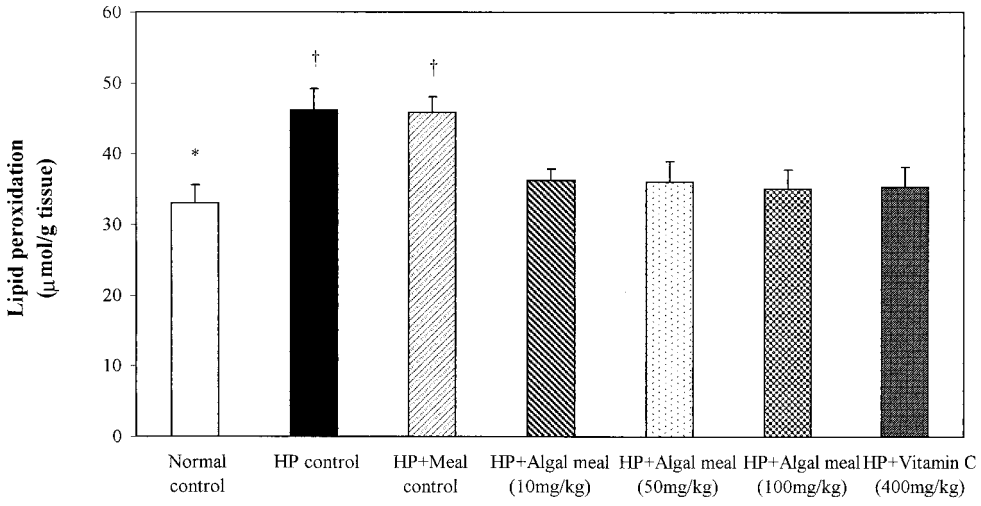


FIG. 4. The concentrations of MDA and 4-hydroxyalkenals in murine stomachs were significantly increased in *H. pylori*-infected untreated and control-meal-treated mice compared to those in normal control animals (*, $P < 0.01$). All antioxidant-treated mice showed significant decreases in lipid peroxidation compared to the levels in untreated and control-meal-treated mice († $P < 0.05$).

sue damage induced by ROMs, and it may also inhibit infection through an altered immune response. As early as the 1930s it was discovered that β-carotene increases our natural resistance to bacterial and viral infections and it was proposed that vitamin A causes this effect (3). It is now well known that other carotenoids also improve the immune defense, and in comparative studies, astaxanthin was shown to be most effective (12, 13). Several studies have shown that strong T helper 1 (Th1) cellular immune responses contribute to *Helicobacter*-associated gastritis and that Th2 T lymphocytes producing interleukin 4 reduce the bacterial load of *H. felis*-infected mice (18, 29). We found recently that astaxanthin-rich algal meal induces a shift of the Th1-Th2 lymphocyte balance associated with an increased natural defense against *H. pylori* infection in this mouse model (1).

In conclusion, our results suggest that the use of antioxidants to combat *H. pylori* infection in humans is an attractive new treatment strategy. Further studies with different feeding formulas and delivery systems as well as prophylaxis studies of animal models and clinical studies are now in progress.

ACKNOWLEDGMENTS

This study was supported by grants from AstaCarotene AB in Gustavsberg, Sweden; the Swedish Medical Research Council (16X04723); the Swedish Forestry Agriculture Research Council (50.0497/98); the Kungliga fysiografiska sällskapet in Lund, Sweden; and the Medical Faculty, Lund University, Lund, Sweden.

REFERENCES

1. **Bennedsen, M., X. Wang, R. Willén, T. Wadström, and L. P. Andersen.** 1999. Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. Immunol. Lett. **70:**185–189.
2. **Buiatti, E., D. Palli, A. Decarli, D. Amadori, C. Avellini, S. Bianchi, C. Bonaguri, F. Cipriani, P. Cocco, A. Giacosa, et al.** 1990. A case-control study of gastric cancer and diet in Italy. II. Association with nutrients. Int. J. Cancer **45:**896–901.
3. **Chew, B. P.** 1993. Role of carotenoids in the immune response. J. Dairy Sci. **76:**2804–2811.
4. **Cohen, M., and H. N. Bhagavan.** 1995. Ascorbic acid and gastrointestinal cancer. J. Am. Coll. Nutr. **14:**565–578.
5. **Correa, P., G. Malcom, B. Schmidt, E. Fontham, B. Ruiz, J. C. Bravo, L. E. Bravo, G. Zarama, and J. L. Realpe.** 1998. Review article: antioxidant micronutrients and gastric cancer. Aliment. Pharmacol. Ther. **12**(Suppl. 1)**:**73–82.
6. **Drake, I. M., M. J. Davies, N. P. Mapstone, M. F. Dixon, C. J. Schorah, K. L. White, D. M. Chalmers, and A. T. Axon.** 1996. Ascorbic acid may protect against human gastric cancer by scavenging mucosal oxygen radicals. Carcinogenesis **17:**559–562.
7. **Ernst, P.** 1999. Review article: the role of inflammation in the pathogenesis of gastric cancer. Aliment. Pharmacol. Ther. **13**(Suppl. 1)**:**13–18.
8. **Fan, L., A. Vonshak, A. Zarka, and S. Boussiba.** 1998. Does astaxanthin protect *Haematococcus* against light damage? Z. Naturforsch Sect. C **53:**93–100.
9. **Goldstone, A. R., P. Quirke, and M. F. Dixon.** 1996. *Helicobacter pylori* infection and gastric cancer. J. Pathol. **179:**129–137.
10. **Goodman, K. J., P. Correa, A. H. J. Tengana, J. P. DeLany, and T. Collazos.** 1997. Nutritional factors and *Helicobacter pylori* infections in Colombian children. J. Pediatr. Gastroenterol. **25:**507–515.
11. **Jarosz, M., J. Dzieniszewski, E. Dabrowska-Ufniarz, M. Wartanowicz, S. Ziemlanski, and P. I. Reed.** 1998. Effects of high dose vitamin C treatment on *Helicobacter pylori* infection and total vitamin C concentration in gastric juice. Eur. J. Cancer Prev. **7:**449–454.
12. **Jyonouchi, H., S. Sun, M. Mizokami, and M. D. Gross.** 1996. Effects of various carotenoids on cloned, effector-stage T-helper cell activity. Nutr. Cancer **26:**313–324.
13. **Jyonouchi, H., L. Zhang, M. Gross, and Y. Tomita.** 1994. Immunomodulating actions of carotenoids: enhancement of *in vivo* and *in vitro* antibody production to T-dependent antigens. Nutr. Cancer **21:**47–58.
14. **Khaled, M. A., and S. A. Sarker.** 1998. Changes of oxidant and antioxidant status in humans due to *H. pylori* infection. Nutr. Res. **18:**1463–1468.
15. **Krinsky, N. I.** 1989. Antioxidant function of carotenoids. Free Radic. Biol. Med. **7:**617–635.
16. **Kurashige, M., E. Okimasu, M. Inoue, and K. Utsumi.** 1990. Inhibition of oxidative injury of biological membranes by astaxanthin. Physiol. Chem. Physics Med. NMR **22:**27–38.
17. **Levine, M.** 1986. New concepts in the biology and biochemistry of ascorbic acid. N. Engl. J. Med. **314:**892–902.
18. **Mohammadi, M., J. Nedrud, R. Redline, N. Lycke, and S. J. Czinn.** 1997. Murine CD4 T-cell response to *Helicobacter* infection: TH1 cells enhance gastritis and TH2 cells reduce bacterial load. Gastroenterology **113:**1848–1857.
19. **Morrow, J. D., B. Frei, A. W. Longmire, J. M. Gaziano, S. M. Lynch, Y. Shyr, W. E. Strauss, J. A. Oates, and L. J. Roberts.** 1995. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. N. Engl. J. Med. **332:**1198–1203.
20. **Nilsson, H. O., P. Aleljung, I. Nilsson, T. Tyszkiewicz, and T. Wadström.** 1996. Immunomagnetic bead enrichment and PCR for detection of *Helicobacter pylori* in human stools. FEMS Immunol. Med. Microbiol. **27:**73–79.
21. **Noroozi, M., W. J. Angerson, and M. E. Lean.** 1998. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. Am. J. Clin. Nutr. **67:**1210–1218.
22. **Ohta, R., N. Yamada, H. Kaneko, K. Ishikawa, H. Fukuda, T. Fujino, and A. Suzuki.** 1999. In vitro inhibition of the growth of *Helicobacter pylori* by oil-macerated garlic constituents. Antimicrob. Agents Chemother. **43:**1811–1812. (Letter.)
23. **Okai, Y., and K. Higashi-Okai.** 1996. Possible immunomodulating activities

of carotenoids in *in vitro* cell culture experiments. Int. J. Immunopharmacol. **18:**753–758.
24. **Palozza, P., and N. I. Krinsky.** 1992. Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. Arch. Biochem. Biophys. **297:** 291–295.
25. **Pettersson, A., and Å. Lignell.** 1999. Astaxanthin deficiency in eggs and fry of Baltic salmon (*Salmo salar*) with the M74 syndrome. Ambio **28:**43–46.
26. **Phull, P. S., C. J. Green, and M. R. Jacyna.** 1995. A radical view of the stomach: the role of oxygen-derived free radicals and antioxidants in gastroduodenal disease. Eur. J. Gastroenterol. Hepatol. **7:**265–274.
27. **Rebouche, C. J.** 1991. Ascorbic acid and carnitine biosynthesis. Am. J. Clin. Nutr. **54**(Suppl. 6)**:**1147S–1152S.
28. **Shinchi, K., H. Ishii, K. Imanishi, and S. Kono.** 1997. Relationship of cigarette smoking, alcohol use and dietary habits with *Helicobacter pylori* infection in Japanese men. Scand. J. Gastroenterol. **32:**651–655.
29. **Sommer, F., G. Faller, P. Konturek, T. Kirchner, E. G. Hahn, J. Zeus, M. Rollinghoff, and M. Lohoff.** 1998. Antrum- and corpus mucosa-infiltrating $CD4^+$ lymphocytes in *Helicobacter pylori* gastritis display a Th1 phenotype. Infect. Immun. **66:**5543–5546.
30. **Stahelin, H. B., K. F. Gey, M. Eichholzer, and E. Luden.** 1991. Beta-carotene and cancer prevention. Am. J. Clin. Nutr. **53:**265S–269S.
31. **Suzuki, H., M. Mori, K. Seto, A. Kai, C. Kawaguchi, M. Suzuki, M. Suematsu, T. Yoneta, S. Miura, and H. Ishii.** 1999. *Helicobacter pylori*-associated gastric pro- and antioxidant formation in Mongolian gerbils. Free Radic. Biol. Med. **26:**679–684.
32. **Tanaka, T., H. Makita, M. Ohnishi, H. Mori, K. Satoh, and A. Hara.** 1995. Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. Cancer Res. **55:**4059–4064.
33. **Tsugane, S., Y. Tei, T. Takahashi, S. Watanabe, and K. Sugano.** 1994. Salty food intake and risk of *Helicobacter pylori* infection. Jpn. J. Cancer Res. **85:**474–478.
34. **Wang, X., H. Sjunnesson, E. Sturegård, T. Wadström, R. Willén, and P. Aleljung.** 1998. Dietary factors influence the recovery rates of *Helicobacter pylori* in a BALB/cA mouse model. Zentbl. Bakteriol. **288:**195–205.
35. **Wang, X., E. Sturegård, R. Rupar, H. O. Nilsson, P. A. Aleljung, B. Carlen, R. Willen, and T. Wadström.** 1997. Infection of BALB/cA mice by spiral and coccoid forms of *Helicobacter pylori*. J. Med. Microbiol. **46:**657–663.
36. **Wang, X., R. Willén, T. Wadström, and P. Aleljung.** 1998. RAPD-PCR, histopathological and serological analysis of four mouse strains infected with multiple strains of *Helicobacter pylori*. Microb. Ecol. Health Dis. **10:**148–154.
37. **Waring, A. J., I. M. Drake, C. J. Schorah, K. L. White, D. A. Lynch, A. T. Axon, and M. F. Dixon.** 1996. Ascorbic acid and total vitamin C concentrations in plasma, gastric juice, and gastrointestinal mucosa: effects of gastritis and oral supplementation. Gut **38:**171–176.
38. **Warren, J. R., and B. Marshall.** 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet **i:**1273–1275.
39. **Zhang, H. M., N. Wakisaka, O. Maeda, and T. Yamamoto.** 1997. Vitamin C inhibits the growth of a bacterial risk factor for gastric carcinoma: *Helicobacter pylori*. Cancer **80:**1897–1903.

ELSEVIER

Immunology Letters 70 (1999) 185–189

Immunology Letters

www.elsevier.com/locate/

VI

Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes

Mads Bennedsen [a,*], Xin Wang [b], Roger Willén [c], Torkel Wadström [b], Leif Percival Andersen [a]

[a] *Department of Clinical Microbiology 9301, Rigshospitalet, Juliane Mariesvej 22, DK-2100 Copenhagen, Denmark*
[b] *Department of Infectious Diseases & Medical Microbiology, University of Lund, Lund, Sweden*
[c] *Sahlgrenska University Hospital, Gothenburg, Sweden*

Accepted 18 August 1999

Abstract

Helicobacter pylori is a Gram-negative bacterium affecting about half of the world population, causing chronic gastritis type B dominated by activated phagocytes. In some patients the disease evolves into gastric ulcer, duodenal ulcer, gastric cancer or MALT lymphoma. The pathogenesis is in part caused by the immunological response. In mouse models and in human disease, the mucosal immune response is characterized by activated phagocytes. Mucosal T-lymphocytes are producing IFN-γ thus increasing mucosal inflammation and mucosal damage. A low dietary intake of antioxidants such as carotenoids and vitamin C may be an important factor for acquisition of *H. pylori* by humans. Dietary antioxidants may also affect both acquisition of the infection and the bacterial load of *H. pylori* infected mice. Antioxidants, including carotenoids, have anti-inflammatory effects. The aim of the present study was to investigate whether dietary antoxidant induced modulation of *H. pylori* in mice affected the cytokines produced by *H. pylori* specific T-cells. We found that treatment of *H. pylori* infected mice with an algal cell extract containing the antioxidant astaxanthin reduces bacterial load and gastric inflammation. These changes are associated with a shift of the T-lymphocyte response from a predominant Th1-response dominated by IFN-γ to a Th1/Th2-response with IFN-γ and IL-4. To our knowledge, a switch from a Th1-response to a mixed Th1/Th2-response during an ongoing infection has not been reported previously.

Keywords: *Helicobacter pylori*; Astaxanthin; Antioxidant; T-cell; Th1; Th2; Interferon; IL-4; Cytokines; Balb/cA mice

1. Introduction

Helicobacter pylori is a Gram-negative pathogen colonizing the human gastric epithelium, causing type B gastritis, peptic ulcer disease and gastric cancer [1]. The pathogenesis of this infection is partly due to the immunological response. In the infected gastric mucosa of mice and humans, the immune response is polarized to a Th1 cell-mediated response with release of IFN-γ, which activates phagocytic cell and contributes to mucosal damage [2–6]. A predominant Th1 response has also been shown in *Helicobacter* infections of inbred mice, often using *Helicobacter felis*, a feline gastric *Helicobacter* more easily infecting mice [7,8]. An adoptive transfer of T-helper-2 (Th2) T-lymphocytes producing IL-4 to mice subsequently infected with *H. felis* infected mice leads to a reduction of the bacterial load and gastric inflammation, whereas an adoptive transfer of Th1 cells increased gastric inflammation and bacterial load of subsequently infected animal [7]. Oral immunization of *H. felis*-infected mice with recombinant urease and cholera toxin induced a shift of T-lymphocyte response from a polarized Th1-response to a mixed Th1/Th2 response and cured the infection [8]. These findings suggest that a polarized Th1-response is associated with the pathogenesis of these gastric diseases and that a Th2 response is associated with control of *H. pylori*/*H. felis* infection.

* Corresponding author. Tel.: +45-3545-4977; fax: +45-3545-6412.
E-mail address: mbe@biobase.dk (M. Bennedsen)

0165-2478/99/$ - see front matter
PII: S0165-2478(99)00145-5

A low dietary intake of antioxidants such as carotenoids and vitamin C has been suggested as an important factor for acquisition of *H. pylori* by humans [9,10]. Dietary antioxidants also affect both acquisition of the infection and the bacterial load of *H. pylori* infected mice [11]. The algae *Haematococcus pluvialis* is rich in astaxanthin, a carotenoid with strong anti-oxidative and anti-inflammatory properties [12,13]. Astaxanthin has a number of immuno-modulatory effects in vitro: (i) enhancement of T-cell dependent antibody production by mouse T-lymphocyte clones and unprimed T-lymphocytes; (ii) enhancement of TNF-α and IL-1a release of mouse peritoneal adherent cells and down-regulation of IFN-release by mouse Th1 clones and primed spleen cells [14–16]. The present study reports the reduction of bacterial load, the decreased gastric inflammation and the changes of the splenocyte cytokine release in response to *H. pylori* antigens induced by feeding *H. pylori*-infected Balb/cA mice with an algal meal containing 2–3% astaxanthin, as compared to infected, untreated mice and uninfected untreated mice.

2. Materials and methods

2.1. Mouse experiments

Twenty Balb/cA mice (B&K Universal Company, Stockholm, Sweden), 6–8 weeks old, were inoculated orally with 10^8 colony forming units (CFU) of the mouse passaged *H. pylori* strain 119/95p three times at two day intervals and ten mice were inoculated with PBS [11]. Two weeks after inoculation, half of the infected mice were treated orally with a cell extract from the micro algae *H. pluvialis* containing the antioxidant astaxanthin (200 mg per kg body weight per day) (AstaCarotene AB, Gustavsberg, Sweden) for 10 days. All mice were sacrificed at the end of treatment. Duodenum, stomach, spleen and blood were removed and prepared for further analysis [11,17].

2.2. Bacterial load

The stomach was opened through the longer curvature and one third was rinsed in sterile phosphate buffered saline (PBS pH 7.2), smeared on GAB-camp agar plates and incubated microaerobic at 37°C for 5–10 days [18]. *H. pylori* was identified as small translucent colonies containing gram negative curved rods, which were urease, catalase and oxidase positive. The identification was confirmed by PCR of the 16S rRNA. The number of *H. pylori* was estimated by colony count.

2.3. Histopathology

The degree of inflammation in antrum and corpus was assessed blindly by a trained pathologist. The scoring system was: 0, normal epithelium; 1, few inflammatory cells; 2, moderate amount of inflammatory cells in several layers; 3, high level of inflammation with nests containing more than 50 inflammatory cells. The grade of gastritis was expressed as mean of antrum, corpus and duodenum scores for each mouse [18].

2.4. Isolation of splenocytes

Splenocytes were washed out of the spleens with RPMI 1640 using a syringe equipped with a 27G needle. Red blood cells were lysed with 0.17 M ammonium chloride and the remaining splenocytes were washed and re-suspended to a concentration of 4×10^7 ml^{-1} in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, Hepes, penicillin and streptomycin.

2.5. Proliferation assay

A number of 400 000 splenocytes were added to each well in 96-well cell culture plates (Nunc, Roskilde, Denmark) and stimulated with 50 μg ml^{-1} heat inactivated *H. pylori* sonicate (Strain CH-20429) in RPMI 1640. The plates were incubated at 5% CO_2 for 36 h at 37° C with ^{3}H-thymidine. Splenocytes in control wells were stimulated with RPMI 1640. The cells were harvested onto a glass fibre filter (Packard, Meriden, CT) and proliferation was measured as ^{3}H-thymidine incorporation in the cells by scintillation counts (kcpm).

2.6. Cytokine release

Four hundred thousand splenocytes were added to each well in 96-well cell culture plates and stimulated medium, 50 μg ml^{-1} *H. pylori* sonicate or 50 μg ml^{-1} heat-inactivated *H. pylori* CH-20429 sonicate and incubated as above. Culture supernatants were harvested after 36 h and stored at −80°C until use. IFN-γ and IL-4 concentration were measured by enzyme linked immunosorbent assay (ELISA) according to the instruction of the manufacturer (DuoSeT, Genzyme, Cambridge, MA). Results were calculated as the difference between cytokine release after antigen re-stimulation and spontaneous cytokine release in medium of cells from the same spleen.

Statistical analysis (*t*-tests) were performed using STATVIEW 4.5 software (Abacus Concepts Inc., Berkeley, USA). The level of significance was selected at $P \leq 0.05$.

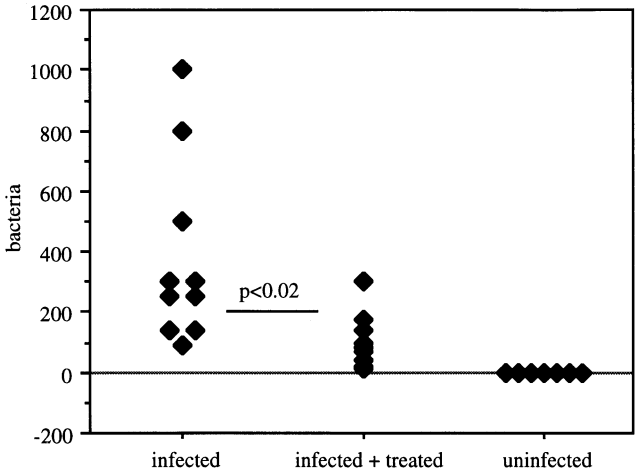


Fig. 1. Reduction in *H. pylori* colonization of gastric mucosa in *H. pylori* infected Balb/cA mice treated with astaxanthin at 3.5 weeks post inoculation as compared to infected, untreated control mice ($P < 0.02$, *t*-test).

3. Results

3.1. Murine gastritis and bacterial load

Inoculation of Balb/cA mice with *H. pylori* resulted in stable infections of the gastric mucosa characterized by a moderate inflammatory response [11,18]. Treatment of the infected mice with an algal cell extract containing astaxanthin reduced bacterial load of gastric mucosa in infected mice as compared to untreated, infected mice (mean CFU 101 versus 407, $P < 0.02$) (Fig. 1). Also the gastritis score of infected mice was reduced after treatment with astaxanthin (mean gastritis score 1.85 versus 1.25, $P = 0.01$) (Fig. 2).

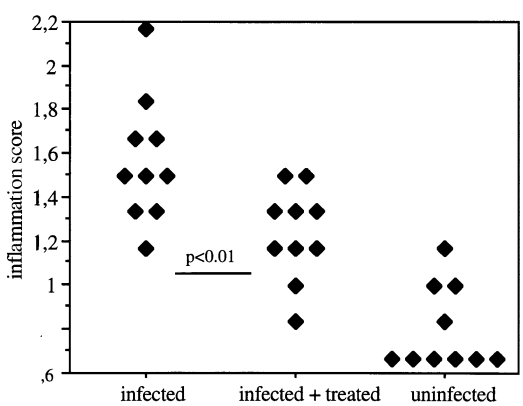


Fig. 2. Reduction in inflammation score of gastritis in *H. pylori* infected Balb/cA mice treated with astaxanthin at 3.5 weeks post inoculation as compared to infected, untreated mice ($P < 0.01$, *t*-test) and uninfected untreated control mice.

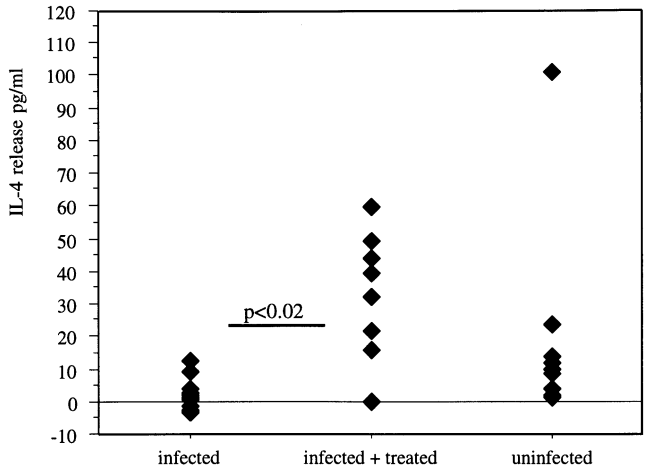


Fig. 3. In vitro induction of IL-4 release of splenocytes from *H. pylori* infected Balb/cA mice treated with astaxanthin at 3.5 weeks post inoculation as compared to infected, untreated mice ($P < 0.01$, *t*-test) and uninfected untreated control mice. The splenocytes were stimulated with 50 mg ml^{-1} of *H. pylori* CH-20429 sonicate or cell culture medium alone. Results are presented as cytokine release after antigen stimulation minus spontaneous release in medium of splenocytes from the same mouse. Ten mice were examined in each group.

3.2. Cytokine release

Splenocytes from all groups of mice proliferated when stimulated with *H. pylori* antigens. Both untreated *H. pylori* infected mice and infected mice treated with astaxanthin released cytokines when stimulated with 50 μg ml^{-1} of *H. pylori* CH-20429 sonicate. Splenocytes from uninfected mice produced only small amounts of IL-4 and IFN-γ. IL-4 release from splenocytes stimulated with the bacterial sonicate was increased in the astaxanthin treated mice as compared to the infected untreated mice (mean release 29.8 pg ml^{-1} versus 2.4 pg ml^{-1}, $P = 0.0002$) (Fig. 3). IFN-γ release from splenocytes stimulated with heat inactivated *H. pylori* sonicate was reduced in the astaxanthin treated mice ($N = 5$ mice) as compared to the infected mice ($N = 5$ mice) (Fig. 4). The difference was not statistically significant.

4. Discussion

In this study we demonstrate, that *H. pylori* infected Balb/cA mice treated with the antioxidant astaxanthin show: (i) a decreased bacterial load and a decreased grade of inflammation in the stomach; (ii) the treatment was associated with a shift in the cytokine release profile of cultured splenocytes in vitro. Balb/cA mice infected with *H. pylori* display a Th1 T-cell response as described in man [2–5]. However, splenocytes from astaxanthin treated animals showed a significant increase in IL-4 release, indicating a shift towards a Th2

T-cell response. An excessive Th1 response driven by the infection with *H. pylori* may favor the development of a cell mediated immune response and may lead to cytotoxic damage of the epithelium [5]. Our data support the notion, that an immunomodulation can enhance a protective and nondestructive response against *H. pylori*, attenuating the infection and the subsequent gastric inflammation [7]. The observed shift of the Th1/Th2-balance following treatment is probably as a result of the down-regulation of Th1-cells and up-regulation of Th2-cells by astaxanthin as previously described [15]. A shift from a Th2 response towards a mixed T-lymphocyte response or a Th1 response is occuring naturally in cutaneous leishmaniasis [19]. In patients suffering from severe cutaneous leishmaniasis isolated T-lymphocyte clones produce predominantly Th2-cytokines. This disease is self-limiting, and during recovery the isolated T-lymphocyte clones are predominantly of Th1 type. The switch towards a Th1 type of response has also been observed after treatment of both cutaneous and visceral leishmaniasis with pentostam. A reverse clinical picture is seen in malaria infections. The first episode of parasitaemia is controlled by a Th1 response, later the immune response shifts and the following episodes are controlled by a Th2 type of response [20]. In the mouse model presented here, the switch from a Th1 type of response to a mixed Th1/Th2 type of response is experimentally induced during an ongoing infection. To our knowledge, it is the first time a shift in T-lymphocyte response has been achieved during an ongoing infection.

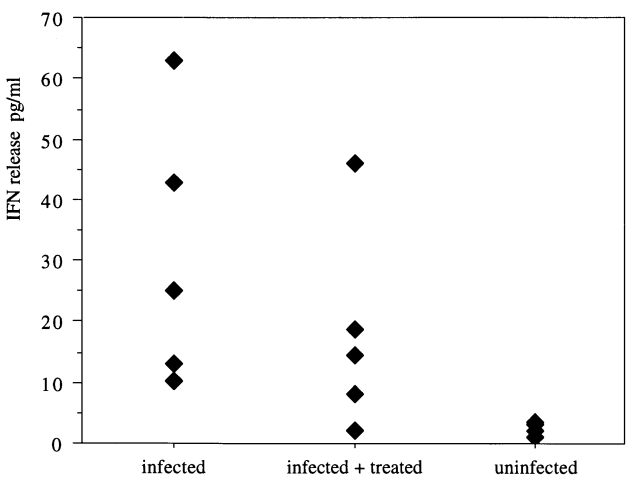


Fig. 4. In vitro IFN-γ release of splenocytes from *H. pylori* infected Balb/cA mice treated with astaxanthin at 3.5 weeks post inoculation as compared to infected, untreated mice and uninfected untreated control mice. The splenocytes were re-stimulated with 50 mg ml^{-1} of *H. pylori* CH-20429 sonicate or cell culture medium alone. Results are presented as cytokine release after antigen stimulation minus spontaneous release in medium of splenocytes from the same mouse. No statistically significant difference was seen between the infected, treated and the infected untreated groups of mice. Five mice were examined in each group.

Another possible mechanism of action is that the antioxidant astaxanthin neutralised reactive free oxygen metabolites (ROM's) in the mucosa and may have attenuated the inflammation. *H. pylori* induces a chronic inflammation dominated by a mixture of neutrophils, monocytes and lymphocytes. It has previously been shown that exposure of human leukocytes to *H. pylori* antigens generates ROM's in vitro [21,22]. Our recent experimental results strongly support the theory, that ROM's may play a part in the pathogenesis of the *H. pylori* induced chronic gastritis.

This study is consistent with the observations showing that an adoptive transfer in mice of Th1 cells enhances gastritis and the bacterial load and that a Th2 cell response reduces these parameters [7] and confirms the protective role of a Th2 cell mediated immune response in *H. pylori* infection.

Goodman et al. [10] reported a three times increased risk of acquisition of *H. pylori* associated with a low intake of dietary carotenoids. Recent preliminary data in a clinical trial of astaxanthin on *H. pylori* infected humans showed clearance of the infection in one out of ten volunteers and reduction of epigastric pain in five of seven volunteers [23]. A randomized double blind clinical trial is necessary to clarify the effects of astaxanthin treatment in *H. pylori* infected humans.

5. Conclusion

Astaxanthin treatment reduced bacterial load and mucosal inflammation in a Balb/cA mouse model of *H. pylori* infection, and that this was associated with a switch in the systemic T-helper cell response. Further studies are needed to describe the effect of astaxanthin on the T-cell reponse in the inflamed gastric mucosa.

Acknowledgements

This study was supported by grants from The Danish Medical Research Council (9601804), Mimi and Victor Larsens Foundation, Direktør Ib Henriksens Foundation, the Swedish Medical Research Council (16X04723) and AstaCarotene AB, Gustavsberg, Sweden.

References

[1] B.E. Dunn, H. Cohen, M.J. Blaser, Clin. Microbiol. Rev. 10 (1997) 720–741.
[2] M.M. Walker, J.E. Crabtree, Ann. NY Acad. Sci. 859 (1998) 96–111.
[3] F. Sommer, G. Faller, P. Konturek, T. Kirchner, E.G. Hahn, J. Zeus, M. Rollinghoff, M. Lohoff, Infect. Immun. 66 (1998) 5543–5546.

[4] C. Lindholm, M. Quiding-Jarbrink, H. Lonroth, A. Hamlet, A.M. Svennerholm, Infect. Immun. 66 (1998) 5964–5971.
[5] S.J. Czinn, J.G. Nedrud, Springer Semin. Immunopathol. 18 (1997) 495–513.
[6] M. Mohammadi, S. Czinn, R. Redline, J. Nedrud, J. Immunol. 156 (1996) 4729–4738.
[7] M. Mohammadi, J. Nedrud, R. Redline, N. Lycke, S.J. Czinn, Gastroenterology 113 (1997) 1848–1857.
[8] P.F. Saldinger, N. Porta, P. Launois, J.A. Louis, G.A. Waanders, H. Bouzourene, P. Michetti, A.L. Blum, I.E. Corthesy-Theulaz, Gastroenterology 115 (1998) 891–897.
[9] E.T. Fontham, B. Ruiz, A. Perez, F. Hunter, P. Correa, Am. J. Gastroenterol. 90 (1995) 1094–1101.
[10] K.J. Goodman, P. Correa, H.J. Tengana-Aux, J.P. DeLany, T. Collazos, J. Pediatr. Gastroenterol. Nutr. 25 (1997) 507–515.
[11] X. Wang, H. Sjunnesson, E. Sturegard, T. Wadstrom, R. Willen, P. Aleljung, Zentralbl Bakteriol 288 (1998) 195–205.
[12] N.I. Krinsky, Free Radic. Biol. Med. 7 (1989) 617–635.
[13] A. Mortensen, L.H. Skibsted, J. Sampson, C. Rice-Evans, S.A. Everett, Febs. Lett. 418 (1997) 91–97.
[14] Y. Okai, K. Higashi-Okai, Int. J. Immunopharmacol. 18 (1996) 753–758.
[15] H. Jyonouchi, S. Sun, M. Mizokami, M.D. Gross, Nutr. Cancer 26 (1996) 313–324.
[16] H. Jyonouchi, S. Sun, Y. Tomita, M.D. Gross, J. Nutr. 125 (1995) 2483–2492.
[17] X. Wang, E. Sturegard, R. Rupar, H.O. Nilsson, P.A. Aleljung, B. Carlen, R. Willen, T. Wadstrom, J. Med. Microbiol. 46 (1997) 657–663.
[18] X. Wang, R. Willén, T. Wadström, P. Aleljung, Micro. Ecol. Health Dis. 10 (1998) 148–154.
[19] M. Kemp, T.G. Theander, A. Kharazmi, Immunol. Today 17 (1996) 13–16.
[20] A.W. Taylor-Robinson, R.S. Phillips, A. Severn, S. Moncada, F.Y. Liew, Science 260 (1993) 1931–1934.
[21] H. Nielsen, L.P. Andersen, Gastroenterology 103 (1992) 1747–1753.
[22] A. Norgaard, H. Nielsen, L.P. Andersen, Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis. 280 (1993) 86–92.
[23] Lignell, Å., R. Surace, P. Böttiger, T. Borody, Symptom improvement in *Helicobacter pylori*-positive non-ulcer dyspeptic patients after treatment with the carotenoid astaxanthin. In: Proceedings of the 12th International Carotenoid Symposium. 1999. Cairns, Australia.