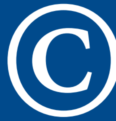

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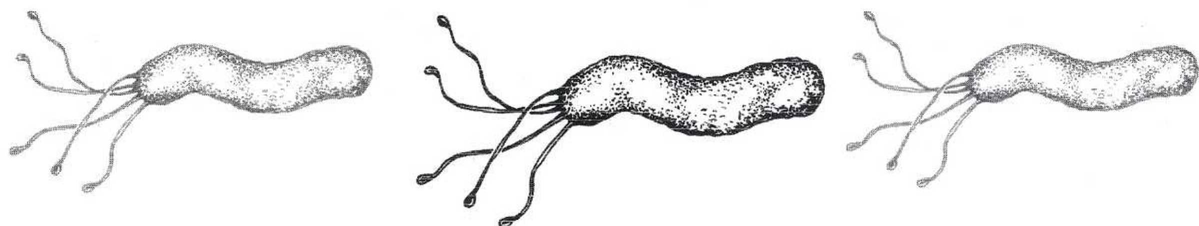
Experimental studies of *Helicobacter pylori* immunization;

Identification of protective immune responses and vaccine candidate antigens



Johanna Nyström

Göteborg 2006

Experimental Studies of *Helicobacter pylori* Immunization; Identification of Protective Immune Responses and Vaccine Candidate Antigens

AKADEMISK AVHANDLING

Som för avläggande av medicine doktorsexamen vid Sahlgrenska Akademin vid Göteborgs Universitet kommer att försvaras offentligt i hörsal "Arvid Carlsson", Academicum, Medicinaregatan 3, Göteborg

Fredag den 19 maj 2006, kl 9.00

av

Johanna Nyström
MSc

Fakultetsopponent:

Professor Erik Kihlström
Linköpings Universitet

Avhandlingen baseras på följande delarbeten:

I Raghavan S, **Nyström J**, Fredriksson M, Holmgren J, Harandi AM.
Orally Administered CpG Oligodeoxynucleotides Induces Production of CXC and CC Chemokines in the Gastric Mucosa and Suppresses Bacterial Colonization in a Mouse Model of *H. pylori* Infection.
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Oral immunization with HpaA affords therapeutic protective immunity against *H. pylori* that is reflected by specific mucosal immune responses
Manuscript

Experimental Studies of *Helicobacter pylori* Immunization;
Identification of Protective Immune Responses and Vaccine Candidate Antigens

Johanna Nyström, Institute of Biomedicine, Göteborg University, 405 30 Göteborg

Helicobacter pylori infection is an important cause of chronic active gastritis, peptic ulcer disease and gastric cancer. The current treatment against *H. pylori*, consisting of a combination of a proton pump inhibitor and two different antibiotics, is effective but has several drawbacks such as high cost and increased risk of antibiotic resistance. A vaccine against *H. pylori* would therefore be an attractive alternative or complement to the current treatment. Using an established mouse model of *H. pylori* infection, we have studied the induction of innate immune responses by CpG oligodeoxynucleotides (ODN). In addition, we sought to identify induction of protection as well as immune responses that are related to vaccine induced protection against *H. pylori*. We also wanted to identify candidate antigens that could be used in a therapeutic *H. pylori* vaccine.
Oral administration of CpG ODN induced local production of chemokines in the gastric mucosa and also resulted in significant protection, i.e. reduction in the bacterial load in the stomachs of *H. pylori* infected mice.
To evaluate protection induced by therapeutic immunization and protective immune mechanisms that can be related to protection against *H. pylori*, vaccination strategies were used that previously had been shown to be effective for prophylactic immunization. Oral therapeutic immunization with *H. pylori* lysate and cholera toxin (CT) as adjuvant induced significant protection, whereas intraperitoneal immunization using lysate and Alum did not induce any such protection. The reduction in bacterial load was related to increased mucosal *H. pylori*-specific Th1-tilted $CD4^+$ T cell responses and to specific IgA levels in saponin-extracted stomach tissue.
H. pylori adhesin A (HpaA) is a surface located *H. pylori*-specific protein present in all strains tested. To evaluate whether HpaA is important for colonization in mice, an isogenic mutant of strain SS1 lacking a functional *hpaA* gene was constructed. To ascertain that disruption of the *hpaA* gene had not introduced any second site mutations or caused any polar effects on its downstream genes, the protein expression patterns of the mutant and the wild-type strain were compared by proteomic approaches. Apart from expression of HpaA, only some minor differences were seen between the two strains and so the mutant strain was tested for its colonizing ability in mice. While inoculation with wild-type *H. pylori* resulted in heavily infected mice, no bacteria could be detected in the stomachs of mice infected with the isogenic HpaA mutant, suggesting that HpaA is essential for colonization of *H. pylori* in mice.
Next, we evaluated the capacity of HpaA to induce protection when administered therapeutically to infected mice. We found that oral immunization with HpaA and CT induced significant protection, which was even more pronounced when a combination of HpaA, urease and CT was used. Similar to the oral immunization with lysate, this protection was also accompanied by antigen-specific mucosal $CD4^+$ T cell responses with a Th1 profile as well as by IgA responses in the stomach.
In conclusion, we have shown that mucosal IgA and $CD4^+$ T cell responses are related to protection against *H. pylori* after therapeutic immunization in mice. In addition, HpaA is a colonization factor for *H. pylori* in mice and a promising candidate antigen for a therapeutic vaccine.

Keywords: *Helicobacter pylori*, Cholera toxin, CpG ODN, $CD4^+$ T cells, IgA, Therapeutic Immunization, HpaA
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Experimental Studies of *Helicobacter pylori* Immunization;
Identification of Protective Immune Responses and Vaccine Candidate Antigens

Johanna Nyström

Institute of Biomedicine
Department of Microbiology and Immunology
Göteborg University
2006

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Drawing of *H. pylori* by Helena Carlsson
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If you only walk in good weather you will
never reach your destination
Anonymous

ABSTRACT

Helicobacter pylori infection is an important cause of chronic active gastritis, peptic ulcer disease and gastric cancer. The current treatment against *H. pylori*, consisting of a combination of a proton pump inhibitor and two different antibiotics, is effective but has several drawbacks such as high cost and increased risk of antibiotic resistance. A vaccine against *H. pylori* would therefore be an attractive alternative or complement to the current treatment. Using an established mouse model of *H. pylori* infection, we have studied the induction of innate immune responses by oral administration of CpG oligodeoxynucleotides (ODN). In addition, we sought to identify induction of protection as well as immune responses that are related to vaccine induced protection against *H. pylori*. We also wanted to identify candidate antigens that could be used in a therapeutic *H. pylori* vaccine.

Oral administration of CpG ODN induced local production of chemokines in the gastric mucosa and also resulted in protection against *H. pylori*, i.e. significant reduction in the bacterial load in the stomachs of *H. pylori* infected mice. The protection correlated with the extent of gastric inflammation and chemokine production.

To evaluate protection induced by therapeutic immunization and protective immune mechanisms that can be related to protection against *H. pylori*, vaccination strategies were used that previously had been shown to be effective for prophylactic immunization. Oral therapeutic immunization with *H. pylori* lysate and cholera toxin (CT) as adjuvant induced significant protection, whereas intraperitoneal immunization using lysate and Alum did not induce any such protection. The reduction in bacterial load was related to increased mucosal *H. pylori*-specific Th1-tilted $CD4^+$ T cell responses and to specific IgA levels in saponin-extracted stomach tissue.

H. pylori adhesin A (HpaA) is a surface located *H. pylori*-specific protein present in all strains tested. To evaluate whether HpaA is important for colonization in mice, an isogenic mutant of strain SS1 lacking a functional *hpaA* gene was constructed. To ascertain that disruption of the *hpaA* gene had not introduced any second site mutations or caused any polar effects on its downstream genes, the protein expression patterns of the mutant and the wild-type strains were compared by proteomic approaches. Apart from expression of HpaA, only some minor differences were seen between the two strains and so the mutant strain was tested for its colonizing ability in mice. While inoculation with wild-type *H. pylori* resulted in heavily infected mice, no bacteria could be detected in the stomachs of mice infected with the isogenic HpaA mutant, suggesting that HpaA is essential for colonization of *H. pylori* in mice. Next, we evaluated the capacity of HpaA to induce protection when administered therapeutically to infected mice. We found that oral immunization with HpaA and CT induced significant protection, which was even more pronounced when a combination of HpaA, urease and CT was used. Similar to the oral immunization with lysate, this protection was also accompanied by antigen-specific mucosal $CD4^+$ T cell responses with a Th1 profile as well as by IgA responses in the stomach.

In conclusion, we have shown that mucosal IgA and $CD4^+$ T cell responses are related to protection against *H. pylori* after therapeutic immunization in mice. In addition, HpaA is a colonization factor for *H. pylori* in mice and a promising candidate antigen for a therapeutic vaccine.

Keywords: *Helicobacter pylori*, Cholera toxin, CpG ODN, CD4+ T cells, IgA, Therapeutic Immunization, HpaA

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV):

I Raghavan S, **Nyström J**, Fredriksson M, Holmgren J, Harandi AM.
Orally Administered CpG Oligodeoxynucleotides Induces Production of CXC and CC Chemokines in the Gastric Mucosa and Suppresses Bacterial Colonization in a Mouse Model of *Helicobacter pylori* Infection.
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Manuscript

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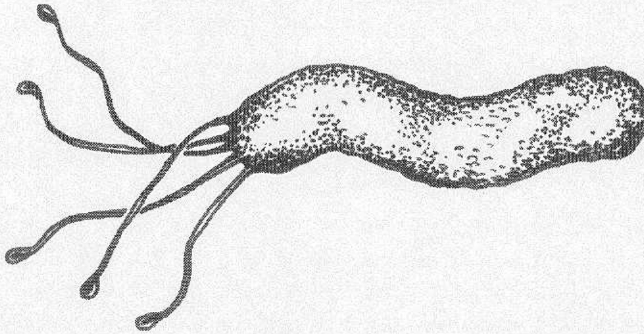
ABBREVIATIONS

1D-GE	One-dimensional gel electrophoresis
2D-GE	Two-dimensional gel electrophoresis
APC	Antigen presenting cell
BabA	Blood group binding adhesin A
CFU	Colony forming units
CpG ODN	Cytidine-phosphate-Guanosine oligodeoxynucleotide
CT	Cholera toxin
CTB	Cholera toxin B subunit
DC	Dendritic cell
DIGE	Differential in-gel electrophoresis
ELISA	Enzyme-linked immunosorbent assay
FT-ICR	Fourier transform-ion cyclotrone resonance
GM	Geometric mean
HpaA	Helicobacter pylori adhesin A
IFN-γ	Interferon- γ
Ig	Immunoglobulin
IL	Interleukin
Ip	Intraperitoneal
IP-10	Gamma interferon-inducible protein 10
Le	Lewis antigen
LPS	Lipopolysaccharide
LT	*E. coli* heat labile enterotoxin
MHC	Major histocompatibility complex
MIP-1a/β	Macrophage inflammatory protein 1 a/β
MLN	Mesenteric lymph node
MS	Mass spectrometry
OMP	Outer membrane protein
RANTES	Regulated on activation, normal T cell expressed and secreted
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SS1	Sydney strain 1
Th	T helper
TLR	Toll like receptor
TNF-α	Tumor Necrosis Factor -α
VacA	Vacuolating cytotoxin A

INTRODUCTION



HISTORY

For a long time, the human stomach was considered to be a sterile organ where no microorganisms could live due to the harsh acidic conditions. However, 1982 Warren and Marshall were able to culture *Helicobacter pylori* bacteria from patients undergoing gastroscopy and found that the bacteria were present in patients with active chronic gastritis, duodenal ulcer or gastric ulcer (1). They were later awarded the Nobel Prize (in 2005) for their discovery of *H. pylori* and its role in gastritis and peptic ulcer disease. The fact that it was a bacterium that caused peptic ulcer and not stress, spicy foods, or other factors was a surprise to the scientific community and Warren and Marshall had a hard time overturning the prevailing dogma. Marshall later fulfilled Koch's postulate by swallowing a solution of the bacteria, thus infecting himself and developed acute gastritis (2). This did not convince the medical world either, and it was not until the early 1990s that it was recognized that *H. pylori* causes gastric ulcer.

HELICOBACTER PYLORI

The gram-negative bacterium *H. pylori* infects the gastric mucosa of more than half of the world's population, making it one of the most common bacterial infections. The prevalence in developing countries can be as high as 80-90%, whereas it is lower in developed countries, ranging between 10-50% (3). While *H. pylori* prevalence decreases in the developed countries (e.g. in North America and Western Europe) no such decrease has been detected in the developing world (4). The prevalence of infection is strongly associated with low socioeconomic status, overcrowding and poor hygiene (4-6). The infection is generally believed to be acquired during childhood (7). Although the mode of transmission has not been completely

elucidated, epidemiological studies suggest that *H. pylori* is primarily spread by the fecal-oral or oral-oral routes (8).

Colonization by *H. pylori* is restricted to the human gastric mucosal cells in the stomach as well as areas of gastric metaplasia in the duodenum. The majority of the infecting *H. pylori* bacteria (90 %) are free-living within the secreted mucus in the stomach. However, a minority of *H. pylori* cells adheres to gastric epithelial cells (9). It has also been suggested that a small number might invade epithelial cells (10) even though this remains controversial. The colonization is thought to be a dynamic process in which the bacterium continuously attaches and detaches from the epithelial cells.

H. pylori can colonize both the antrum and the more acidic corpus parts of the stomach. All *H. pylori* infected individuals develop chronic active gastritis, which is characterized by recruitment of immune cells and epithelial cell damage. Despite this gastritis, most infected individuals remain asymptomatic. However, 15% of the *H. pylori* infected individuals develop duodenal ulcer and about 2% gastric adenocarcinoma (11). In fact, *H. pylori* was designated a class 1 carcinogen in 1994 by the World Health Organization (WHO).

Why do some individuals remain asymptomatic and some develop disease? Similarly to many infectious diseases, the outcome of infection depends on both environmental, host and microbial factors.

The current treatment against *H. pylori* infection consists of a combination therapy with two different antibiotics together with a proton-pump inhibitor, which in most cases results in successful eradication of the bacteria and healing of ulcers (12). However, there are some major drawbacks with such therapy including high cost, poor patient compliance and increased risk of developing antibiotic resistance, making it unsuitable for use e.g. in the developing world (13, 14). Furthermore, such treatment does not protect against reinfections, which frequently occur in areas with high prevalence of *H. pylori* (15-17). A vaccine would therefore be a suitable alternative for control of *H. pylori*. It should preferably work on two different levels, i.e. to result in a decreased risk of developing *H. pylori*-associated disease for an individual, and to decrease the risk of infection at a population level. A prophylactic vaccine would be given before an individual becomes infected with *H. pylori*, i.e. in children. A therapeutic vaccination would primarily be given to those that have developed *H. pylori* associated diseases. In addition, because chronic *H. pylori* infection, even in the absence of symptoms, is a risk factor for development of adenocarcinoma (18), vaccination of asymptomatic carriers may be justified. Furthermore, a therapeutic vaccine would confer protection against reinfection.

VIRULENCE FACTORS FOR COLONIZATION AND INFLAMMATION

In order to establish and maintain infection, *H. pylori* expresses a variety of different types of virulence factors, to enable survival and colonization in the stomach. These factors include the urease enzyme which creates a favourable environment for *H. pylori* in the acidic stomach, the flagellae which provides motility, and different adhesion factors, e.g. BabA and SabA, which enable *H. pylori* to adhere to surface mucosal cells and to components of the mucus layer and thereby avoid bacterial shedding. Some strains are more pathogenic and are associated with more severe disease outcome, due to the virulence factors they possess (Table 1 and Fig. 1).

HpaA

The *H. pylori* adhesin A (HpaA) is a lipoprotein (19) initially described as a sialic acid binding adhesin, but supportive evidence for its adhesion capacity is still lacking (19, 20). HpaA is surface-located (21-23) but has been found to be expressed in even higher quantities in the inner membrane of *H. pylori* and is also present in the cytoplasm (E. Carlsohn, personal communication, 19). *H. pylori* infected individuals mount serum antibodies, but only low levels of mucosal IgA antibodies against HpaA (24, 25). The expression of the HpaA protein is highly conserved among *H. pylori* isolates (24, 26). Indeed, we have not identified any *H. pylori* strain lacking this lipoprotein among several hundred isolates tested. Furthermore, genomic studies (27, 28) do not show any significant sequence homologies or similarities of HpaA with other known proteins. Taken together, this makes HpaA a putative candidate vaccine antigen against *H. pylori* infection.

Table 1. ***H. pylori*** **virulence factors.**

Virulence Factor	Properties	Localization	Colonization factor[a]	Protective antigen
Urease[b]	Neutralizes the acidic environment in the stomach by hydrolyzing urea to ammonia and bicarbonate ions.	Cytoplasm and outer membrane (29)	Yes (30, 31)	Yes (32)
BabA & SabA	Mediate adhesion to epithelial cells by binding to Lewis b and sialyl Lewis x receptors, respectively, on epithelial cells in the gastric mucosa (33-35).	Surface	Not known	Yes (36)
CagA[b] and Cag PAI	CagA is located within the Cag pathogenicity island (Cag PAI) (a 40kB segment containing about 30 genes) and is associated with more severe outcome of disease (37). Many genes in CagPAI encode for type IV secretion system (T4SS) that can deliver Cag A, and possibly other proteins, into the mammalian host and thereby affecting the function of the cell.	Intracellular	No, since not all strains have CagPAI (38, 39).	Yes, against CagA+ strains (40).
VacA	Induces vacuolation of epithelial cells (41) and can block T cell proliferation (42). $CagA^+$ and $VacA^+$ strains are associated with more severe disease outcome.	Secreted	No, since not all strains express VacA.	Yes, against strains expressing VacA (40).
Nap[b]	Attracts neutrophils to the site of infection and induces release of reactive oxygen species by these cells (43).	Intracellular	Not known	Yes (44)
HpaA[b]	Lipoprotein with unknown function	Intracellular and on the outer membrane (23)	Yes (Paper III)	Yes (Paper IV)
LPS	Endotoxic activity, stimulates the immune system to produce proinflammatory cytokines and activate the complement system. Could be implicated in autoimmunity through molecular mimicry and evasion of the immune system by camouflage (45).	Outer membrane	Not known	Not known

[a] My definition of a colonization factor is an antigen which enables establishment of *H. pylori* infection in the gastric mucosa.

[b] These antigens have been further studied in this thesis

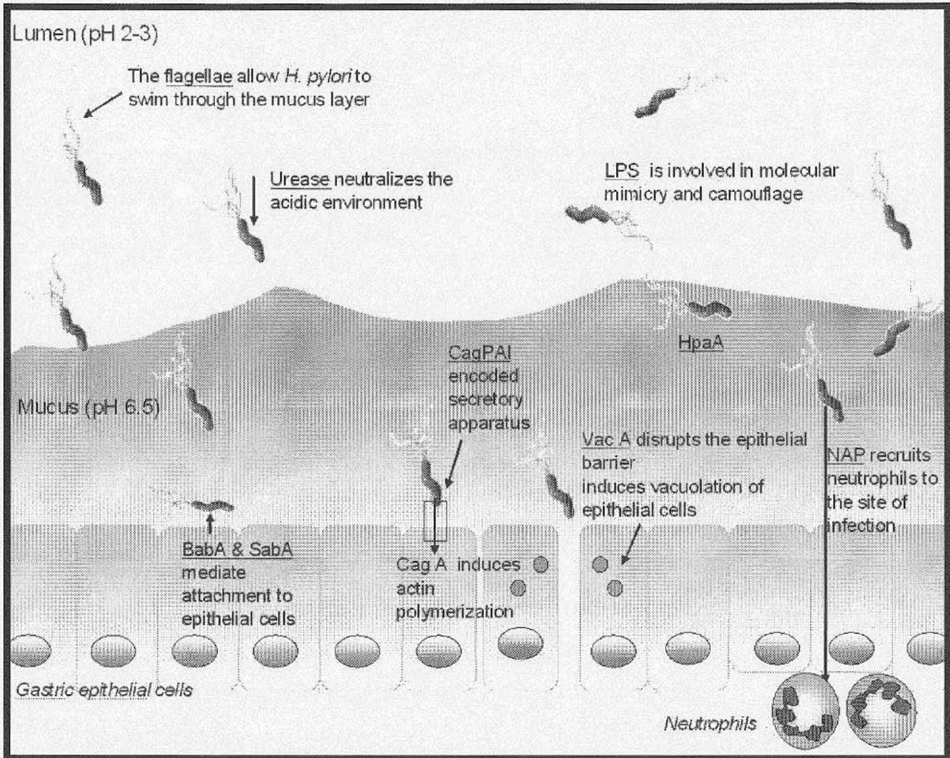


Fig. 1. Some *H. pylori* virulence factors and their roles for colonization and disease in the gastric mucosa.

VACCINATION AGAINST *H. PYLORI*

Vaccination against *H. pylori* would be an effective and cost-effective means of controlling *H. pylori* disease. Because *H. pylori* infection by itself does not lead to spontaneous eradication but rather stays chronic for life, it is tempting to conclude that the immune responses induced by the natural infection is not sufficient *per se* to eradicate *H. pylori*. Also, vaccination with *H. pylori* antigens alone has not conferred any protective immune responses against *H. pylori* in experimental studies (46).Thus, the effectiveness of a vaccine depends on the development of an appropriate antigen formulation that includes an immunostimulatory adjuvant.

Adjuvants

The only approved adjuvants for human use are aluminium salts (aluminium phosphate and aluminium hydroxide), generally called alum, and the new MF59 adjuvant, a squalene oil-in-water emulsion (47). The mechanism by which alum enhances the immune response is not completely known, but it has been suggested that it works by activating antigen presenting cells (APC) e.g. dendritic cells (DC), stimulate the complement system and induce chemokine production (48). Although alum has a good safety record, it is a weak adjuvant for induction of antibodies and cell-mediated immunity against subunit proteins (49). In addition, there are associations between alum and allergic reactions (49). Also, both MF59 and alum are included in parenteral vaccines and are generally not suitable for induction of mucosal immunity. Thus, there is an urgent need for a safe and effective mucosal adjuvant for human use.

Mucosal adjuvants

The primary reason for using mucosal vaccination is to induce an immune response at the site of infection i.e. at the mucosal surface, because most infectious agents enter through or colonize mucosal surfaces. This is true for *H. pylori*, which is not an invasive bacterium.

<u>Cholera toxin (CT) and CT derivatives</u>

Cholera toxin (CT), which is responsible for the severe cholera diarrhea in humans (50), has been extensively used as a potent mucosal adjuvant in several animal studies (51-54). The CT holotoxin consists of two subunits: an enzymatically active/toxigenic A subunit (CTA), and a pentameric B subunit (CTB), which is responsible for binding of CT to the ganglioside GM1 receptor, a glycosphingolipid found on the surface of all nucleated cells, including mucosal epithelial cells (55). Binding to the epithelial cell enables CTA to enter the cytosol, where it ADP ribosylates the stimulatory α-subunit of the GTP-binding protein Gs. This leads to activation of adenylate cyclase which results in increased intracellular cAMP levels in the cell (56, 57).

Oral administration of CT increases the permeability of epithelial cells (58), which can lead to increased uptake of coadministered antigen. However, the most

important contributors to immunity induced by CT are believed to be those leading to increased antigen presentation by DC, B cells and macrophages. Indeed, CT upregulates the expression of MHC molecules and costimulatory molecules e.g. CD80/B7.1 and CD86/B7.2 as well as chemokine receptors on both murine and human APC (59, 60).
CT activates the cellular immune responses to coadministered antigens and increases the induction of mucosal IgA as well as $CD4^+$ and Class I cytotoxic T cell responses (61-63). However, the inherent toxicity of CT precludes its use as an oral adjuvant in humans (64). Indeed, it has been shown that administration of as little as 5 μg CT to human volunteers induces significant diarrhea and that 25 μg may give rise to 20 liter watery diarrhea (65). In contrast, mice may be given 10 μg CT orally without any diarrhea. The nontoxic CTB subunit is a poor adjuvant for induction of mucosal immune responses in mice when given via the oral route, but its adjuvanticity is increased when administered intranasally (66, 67). Another approach to decrease the toxicity of CT but to retain its adjuvanticity has been to link the enzymatically active CTA subunit to the cell binding moiety of *Staphylococcus aureus* protein A (CTA1-DD). In addition, by combining CTA1-DD with immune stimulating complexes (ISCOMS), systemic and mucosal T helper (Th)1 and Th2 responses are shown after oral and intranasal administration (68).

<u>LT and LT derived adjuvants</u>
E. coli heat labile enterotoxin (LT) has 80% homology with CT in the primary structure and they have super-imposable tertiary structures (69, 70). Similarly to CT, LT has been used as a mucosal adjuvant in many animal studies but is too toxic for human use (71, 72).
One approach to circumvent the toxicity of LT has been to construct mutants in the active site of the A subunit, thus inhibiting the ADP ribosylating activity. In the adjuvant LTK63 a serine-to-lysine substitution in position 63 of the A subunit has been introduced. This adjuvant has no enzymatic activity or toxicity in vivo or in vitro, but maintains all the biological properties such as receptor binding (73). LTK63 is a potent mucosal adjuvant, even though its adjuvant activity is lower than that of LT. In addition, LTK63 exhibits better adjuvanticity than LTB showing that even though the A subunit is enzymatically inactive, it plays an important role for the induction of immune responses. Furthermore, immunization using *H. pylori* antigens and LTK63 has conferred protection against *H. pylori* in mice (40, 74) and LTK63 has been tested in humans as a mucosal adjuvant for an influenza vaccine (75).
Another class of detoxified toxin adjuvants is LTR72 (where alanin has been changed to arginine). This adjuvants maintains 1% of the wild-type ADP-ribosylating activity and has been shown to be as effective as LT for induction of immune responses (76).
In addition, the adjuvant LT192, where LT has been altered by one amino acid substitution at the site of proteolytic cleavage, has reduced enterotoxicity (77). However, its adjuvanticity against coadministered antigens is retained, but

unfortunately it was shown to induce diarrhea in humans, at doses as low as 25 μg (78).

CpG

Unmethylated CpG dinucleotides with particular sequence context (CpG motifs), occur at high frequency in microbial DNA and stimulate the mammalian immune system (79). Mammalian immune system appears to have evolved Toll-like receptor 9 (TLR-9) that distinguishes bacterial DNA from self DNA. Interactions between unmethylated CpG motifs in bacterial DNA and TLR9 in APC rapidly activate target cells through the Toll/IL-1-receptor signaling pathway to produce various proinflammatory and Th1-polarizing cytokines and chemokines, up-regulation of co-stimulatory molecules on APC, and activation of B cells for proliferation, IL-6 secretion and antibody production (79-81). The immunostimulatory features of bacterial DNA can be recapitulated by synthetic oligodeoxynucleotide (ODN) containing CpG motifs. The utility of CpG ODN as a Th1-tilting immunostimulator/adjuvant, either singly or in combination with various antigens, for induction of both systemic and mucosal immune responses in experimental animals has been reported (79, 82). In addition, CpG has been tested in humans against different disorders e.g. Hepatitis B (83, 84) cancer (85) and allergy (86), and has been shown to be safe and well-tolerated.

Immunization studies against *H. pylori*

There have been many immunization studies against *H. pylori* infection in mice, using whole cell lysate, or single antigens together with an appropriate adjuvant (for examples, see Table 2). Both prophylactic and therapeutic immunization studies have been performed, although prophylactic studies dominate. Many of these studies have not conferred sterilizing immunity, but 1-2 log (i.e. 10-100 fold) reduction in bacterial load. Vaccine studies against *H. pylori* have been performed in humans, and although mucosal immune responses have been induced by both urease and inactivated whole *H. pylori* bacteria, they have not proven as successful as was hoped for (78, 87). Whether this has been due to use of inappropriate antigens or failure to mount sufficiently high *H. pylori* specific immune responses is not known.

Table 2. Immunization studies against *Helicobacter* in mice using protein antigen and adjuvants.

Antigen	Adjuvant	Prophylactic/ Therapeutic	Route of administration	Ref
Killed *H. pylori*	CT	Prophylactic	Oral	(88)[a]
H. pylori lysate/ inactivated whole-cell *H. pylori*	CT	Therapeutic	Oral	(51, 89)
Urease	LT	Prophylactic	Oral	(90)[a]
Urease/CagA/ *H. pylori* lysate	LTK63	Prophylactic	Oral	(74)
Nap	LTK63	Prophylactic	Oral	(44)
VacA and CagA	LTK63	Therapeutic	Oral	(40)
H. pylori lysate	Alum or Freund's	Prophylactic	Intraperitoneal	(91)
H. felis lysate	CpG + CT	Prophylactic	Intranasal	(92)[a]
H. pylori lysate	CpG	Prophylactic	Intranasal	(93)
H. pylori lysate	CpG	Prophylactic	Subcutaneous	(94)

[a] Mice were challenged with *H. felis*

Protective immune response against *H. pylori*.

There is clear evidence that $CD4^+$ T cells are required for protective immunity against *H. pylori* infection. Immunization of mice lacking MHCI, but not MHCII, are protected against *H. pylori* infection, suggesting that $CD4^+$, but not $CD8^+$ T cells are required for effective immunity (95, 96). In addition, knockout studies have suggested that it is mainly $CD4^+$ T cells of the Th1 type that are responsible for the protective immunity because mice lacking IL-12 or IL-18 cannot mount a protective immune response against *H. pylori* (97, 98).

However, the role of antibodies for immunity against *H. pylori* is less clear. Czinn et al. showed that preincubation of bacteria with urease specific monoclonal antibodies decreased the bacterial infectivity (99). On the other hand, knockout studies have shown that mice lacking antibodies are equally well protected against challenge as wild-type mice (95, 100) and that antibodies actually may impair the gastritis seen, because mice lacking antibodies have more gastritis and also clear the infection quicker than wild-type mice (46). In addition, it has been demonstrated that the prevalence of *H. pylori* infection does not differ between IgA deficient and normal Swedish individuals (101). However, patients with IgA deficiency are at increased risk of developing gastrointestinal carcinomas (102, 103). Also, a recent study shows that gastric adenocarcinoma patients have decreased production of gastric IgA antibodies which may have an impact on the development of disease (Quiding-Järbrink et al., submitted for publication). However, it is not known

whether the low IgA production is a cause or effect of development of gastric malignancies.

It is important to keep in mind that most conclusions regarding protective immune responses against *H. pylori* have been based on results from knock-out studies in mice, where compensatory mechanisms or alternative pathways to achieve immune-mediated protection might occur. It is therefore precautious to assume that $CD8^+$ T cells or antibodies are irrelevant for protection; it could be that the antigens tested have not had the correct antigenic structure to induce the appropriate immune responses. Thus, it may be possible that different immune responses are important against different antigens, e.g. IgA may be effective against one virulence factor whereas cellular immune responses are important against another. Hence, until the mechanisms of immune protection have been further elucidated, it is better to have a vaccine that may induce both cellular and humoral immune responses.

AIMS OF THE STUDY

The overall aims of this thesis were to study protection and related immune responses induced by therapeutic immunization against *H. pylori* in mice and also to identify candidate antigens for possible use in a therapeutic vaccine against *H. pylori*.

The specific aims were:

- To study the induction of innate immunity, i.e. chemokine responses, after oral administration of a putative mucosal adjuvant, CpG ODN, and to investigate its impact on an already established *H. pylori* infection.

- To compare induction of protection by therapeutic immunization with different antigens and by different routes of administration.

- To identify immune responses that are related to protection after vaccination against experimental *H. pylori* infection.

- To establish methods for studies of mucosal T cell responses against *H. pylori*.

- To evaluate the role of a candidate vaccine antigen, HpaA, for colonization of *H. pylori* in mice.

- To identify candidate antigens for a therapeutic vaccine against *H. pylori*.

MATERIAL AND METHODS

H. pylori strains and antigens

H. pylori bacteria used for infection

The mouse adapted strain *H. pylori* strain SS1 (CagA$^+$, VacA$^+$, Le^y) (104) was used in all studies for infecting mice (89). Briefly, the bacteria were grown on Colombia-iso plates for 2-3 days before being transferred to liquid culture for over night culture. About 90% of the bacteria grown under liquid conditions have bacillary form and are motile which is essential for successful infection. The bacteria were resuspended in Brucella broth and adjusted to an OD_{600} of 1.5, and mice were orally infected with $3x10^8$ colony forming units (CFU).

H. pylori antigens

H. pylori strain Hel 305 (CagA$^+$, VacA$^+$, Le^x), a clinical isolate from a Swedish duodenal ulcer patient, was used for preparation of lysate as previously described (40). Plate grown bacteria were harvested in PBS and sonicated on ice before centrifugation and sterile filtration. The lysate was used for immunizing mice (II, IV) and for antigen specific T cell proliferation assays (II).

Membrane preparations (MP) of *H. pylori* were prepared by sonication of bacteria of strain Hel 305 or SS1 followed by differential centrifugation (105). The MP was used as antigen in ELISA for determination of antibody responses against *H. pylori*. Immunoblotting of MP and lysate of strain Hel 305 against some immunodominant antigens (Fig. 2) showed that they contain similar amounts of these antigens.

HpaA, kindly provided by AstraZeneca (Boston, USA), was recombinantly produced and purified, as previously described (106). Urease was purified from *H. pylori* strain E32, which is a good producer of urease (25). *H. pylori* rCag antigen (Austral Biologicals, California, USA) was produced in genetically engineered *E. coli* and covers Glu 748 to Glu 1015 of the *H. pylori* CagA antigen. Nap and catalase were recombinantly produced in *E. coli* (107, 108) and were kindly provided by S. Nyström and C. Cederberg, respectively, AstraZeneca, Sweden.

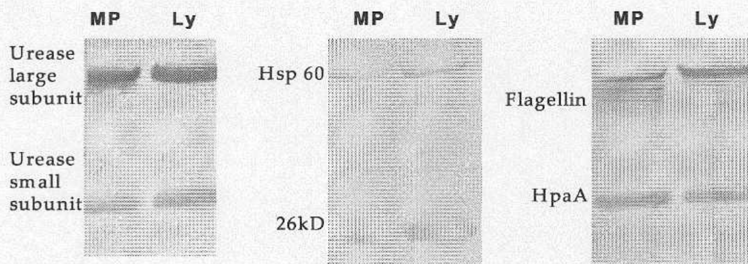


Fig. 2. Membrane (MP) and lysate (Ly) preparations (15 µg) of *H. pylori* strain Hel 305 were blotted against *H. pylori* specific monoclonal antibodies (105, 107). NAP was found in both preparations, but to a lesser degree in MP than in lysate (data not shown).

H. pylori mouse model (Paper I-IV)

The first mouse models of *Helicobacter* used *H. felis* as challenge strain (99) and *H. felis* has been extensively used in further studies (67, 109-111). However, even though this *Helicobacter* model is well established, it has several disadvantages e.g. *H. felis* does not tightly adhere to gastric epithelial cells in vivo and it lacks CagA and VacA which are strongly associated with development of human disease (112). In addition, *H. felis* cannot be quantified by culture from the mouse stomach, making quantitative assessment of protection difficult. Last, but not least *H. felis* and *H. pylori* are different species.

In this thesis, a well established mouse model of *H. pylori* has been used, in which female C57BL/6 mice are infected with the mouse adapted strain, Sydney Strain 1 (SS1), resulting in stable colonization 1-8 weeks post inoculation (51). A recent study has demonstrated that the pattern of *H. pylori* proteins that are expressed in infected mice and become exposed to the mouse immune system appear to be similar to those in human *H. pylori* infections, suggesting that *Helicobacter* models may thus be valid to screen antigens for human vaccination (113).

Therapeutic immunization of *H. pylori* infected mice (Paper II, IV)

The focus of this thesis has been on therapeutic immunization, i.e. immunization of mice with an already established *H. pylori* infection. This was done by immunizing mice two weeks after infection, when the colonization is stable, with *H. pylori* antigen and adjuvant. In initial studies, four weekly doses with *H. pylori* lysate and CT were used for oral immunization, but subsequent studies (II) showed that two immunizations with a two week interval were actually more effective than four weekly doses. Therefore, in most cases the infected mice were orally immunized twice. For parenteral immunization, intraperitoneal (ip) immunization with *H. pylori* antigen and alum was given twice with a two week interval (Fig. 3).

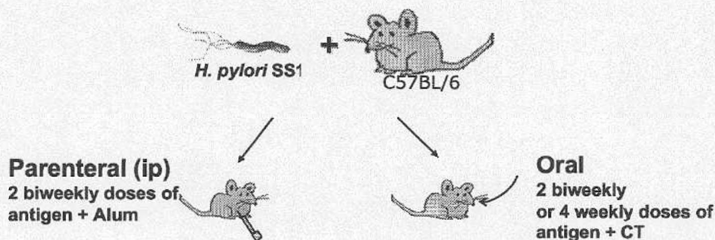


Fig. 3. Experimental set-up for therapeutic immunization studies against *H. pylori* (II, IV).

Determination of bacterial colonization and immune responses induced by infection and therapeutic immunization (I-IV)

For a summary of methods used in this thesis, see Table 3.

Table 3. Methods used for determination of protection and immune responses.

Determination of:	Method	Paper
Bacterial load	Quantitative culture	I-IV
Serum IgG+M antibodies	ELISA[a]	II-IV
Mucosal IgA antibodies in saponin extracts	ELISA[a]	II, IV
T cell responses in MLN	$CD4^+$ T cell proliferation assay	II, IV
Cytokine production	Cytometric bead array, RT-PCR[b]	II, IV
Chemokines in saponin extracts	ELISA[a]	I
Cell infiltration	Hematoxylin and eosin staining	I
Characterization of SS1 (Δ*hpaA*)	DIGE[c], FT-ICR MS[d], RT-PCR[b]	III

[a] ELISA; Enzyme Linked ImmunoSorbent Assay

[b] RT-PCR; Reverse Transcriptase Polymerase Chain Reaction

[c] DIGE; Differential In Gel Electrophoresis

[d] FT-ICR MS; Fourier Transform-Ion Cyclotron Resonance Mass Spectrometry

Determination of *H. pylori* in the stomachs of infected and immunized mice (I-IV)

Several different methods for detection of *H. pylori* bacteria in stomachs of mice have been used, e.g. histology, urease test and culture of stomachs (32, 51, 114). While histology and culture are both quantitative, urease tests are not. In addition, the level of sensitivity for both histological and urease assays is about 10^5 bacteria per gram of tissue (52) and may therefore give false indications of sterilizing immunity after immunization. In contrast, culture of stomachs is a much more sensitive method with a detection limit of about 200 bacteria/stomach. Therefore, the bacterial load of *H. pylori* in the stomachs of infected and immunized mice was determined by quantitative culture of homogenized stomach on *Helicobacter*-selective Skirrow blood agar plates. In cases of uncertainty, the *H. pylori* identity was confirmed by dot-blot staining using a monoclonal antibody against the *H. pylori* specific protein HpaA (26). Protection against *H. pylori* infection was defined as significant reduction in bacterial load compared to in control infected mice ($p<0.05$).

Determination of antibody responses (II-IV)

Serum IgG+IgM

ELISA was used for determination of serum antibody titers against *H. pylori*. The coating antigen used was MP from strain SS1, but coating of the plates with *H. pylori* lysate from Hel 305 gave comparable results (data not shown). We detected both serum immunoglobulin (Ig)G and IgM antibodies against *H. pylori*.

Mucosal IgA

There are different methods to determine mucosal IgA antibodies, e.g. measurement of IgA in fecal pellets or in gut washings (115-117). However, the disadvantage of using these methods is that it is not known where in the gastrointestinal tract the IgA is being produced as it measures IgA in the luminal content. We have therefore used a reproducible and sensitive method for detection of mucosal IgA, e.g. saponin extraction of the mucosal tissues combined with ELISA (118). This method has the advantage of measuring both antibodies in specific compartments of the gastrointestinal tract, e.g. stomach and small intestine. Furthermore, both antibodies that have been secreted and antibodies that are in the tissue can be detected. For the extraction, stomach and small intestines were collected, washed in PBS, weighed and frozen in a PBS solution containing protease inhibitors, i.e. soy trypsin inhibitor and phenylmethylsulfonyl fluoride, as well as EDTA. Saponin was added to the solution to permeabilize the cell membranes. The samples were stored at 4°C overnight. The organs were spun down and the supernatant was analyzed for antibody content by ELISA.

In the original study, mice were perfused in order to remove possible contamination of antibodies from the circulation. However we did not perfuse the mice since we could not detect any differences in *H. pylori* specific IgA titers in sera of immunized and infected mice indicating that it was mucosal IgA that was detected in the saponin extracts and not antibodies transudating from sera. In addition, this saponin extraction method was also used for detection of chemokines in stomach, small intestine and mesenteric lymph nodes (MLN) (paper I).

Determination of mucosal T cell responses (Paper II, IV)

To determine if an immunization regimen against *H. pylori* induces antigen specific T cell responses, spleen cells have been have chosen in many studies (91, 97, 119). However, spleen cells are not suitable for assessing mucosal T cell responses. Indeed, when comparing the proliferative response of spleen cells to that of MLN cells, it was clear that the MLN response better reflected the gastrointestinal responses induced by immunization because oral immunization induced a strong antigen-specific proliferation in cells from MLN but not from spleen (Fig. 4). We have therefore used MLN cells for determination of mucosal T cell responses after therapeutic oral immunization in our studies.

Because $CD4^+$ T cells have been implicated to play a critical role for protection against *H. pylori* (95-97), we sought to specifically evaluate the *H. pylori*-specific $CD4^+$ T cell response in immunized and infected mice.

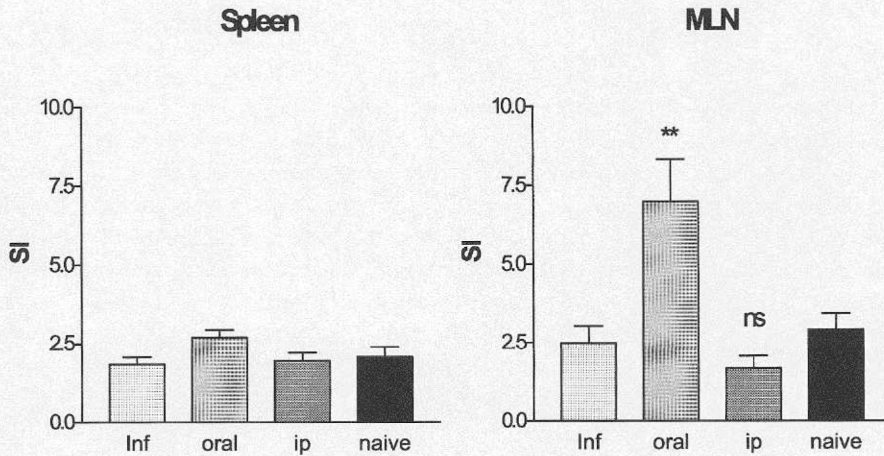


Fig. 4. Antigen-specific proliferation of spleen and MLN cells in infected and immunized mice. Spleen (A) and MLN (B) cell proliferation using cells from control infected and therapeutically immunized mice stimulated with *H. pylori* antigen. Inf; control infected mice (n=8), oral; infected and orally immunized mice (n=8), ip; infected and ip immunized mice (n=6), naïve; neither infected nor immunized mice (n=4). Stimulation index (SI) was determined by calculating the ratio between the proliferation of cells stimulated with antigen and with medium alone. **, $p<0.01$ compared to control infected mice.

Antigen- specific $CD4^+$ T cell proliferation (Paper II, IV)

To determine the antigen-specific $CD4^+$ T cell responses, we first started by simply coculturing $CD4^+$ T cells purified from MLN with *H. pylori* lysate and antigen presenting cells. However, the antigen seemed to be toxic to the isolated T cells, because these cells died by the antigen stimulation. We therefore established a method to circumvent the toxicity. In this method, either B cells or DC were used as APC and were pulsed with *H. pylori*-antigen over night, and any unprocessed antigen was washed off before culturing the cells together with purified $CD4^+$ T cells (Fig. 5). Supernatants were taken after 48 and 96 h for determination of cytokines by cytometric bead array (CBA). The proliferation of cells was determined after four days of culture and measured by thymidine incorporation.

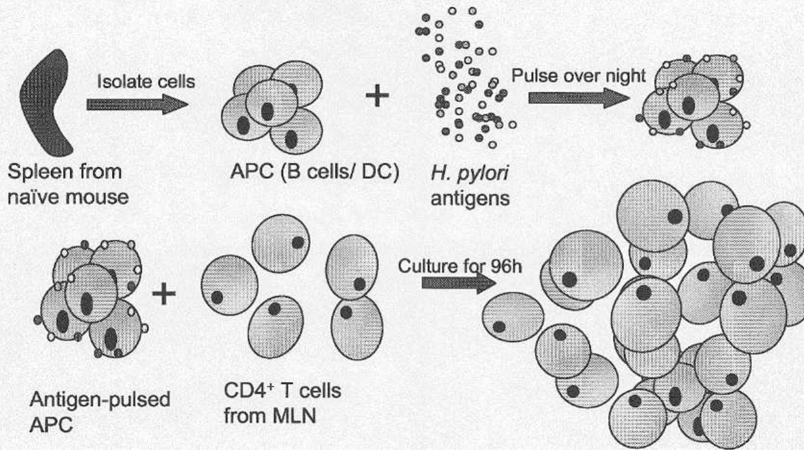


Fig. 5. Schematic picture of the antigen-specific CD4+ T cell proliferation assay.

Proteomics (Paper III)

In paper III we used an SS1 (Δ*hpaA*) mutant to study the physiological role of HpaA for *H. pylori* colonization in mice. To ascertain that disruption of the *hpaA* gene had not introduced any second site mutations or caused any polar effects on its downstream genes, the protein expression patterns of the mutant and the wild-type strains were compared by a proteomic approach since it is a convenient method of monitoring changes in protein expression without prior knowledge of what those changes might be.

Two-dimensional gel electrophoresis (2D-GE), in which proteins are separated both dependent on their molecular weights and isoelectric points, is a powerful method for separation of complex protein mixtures. However, one disadvantage with this method is the problem of reproducibility between different gels.

Thus, differential in gel electrophoresis (DIGE) was used to compare the overall protein expression of the wild-type and mutant strains. In this method, two sets of protein mixtures are run in the same gel, which minimizes the problems with reproducibility. By labelling the different samples with fluorescent dyes, the protein profile of each sample can easily be imaged by fluorescent excitation of the different dyes. In addition, an internal standard is co-run together with the samples which enables a comparison between different gels. In this study, lysates of the wild-type and mutant strain were compared using DIGE analysis. By the use of a computer program, the protein expression of the wild-type and mutant strains could be determined. Protein spots that were significantly altered ($p<0.05$) were selected for identification by mass spectrometry (MS).

Hydrophobic proteins, e.g. membrane proteins, are known to be discriminated in 2D gels. We therefore compared the outer membrane protein (OMPs) profiles of the wild-type and mutant strains by subcellular fractionation of OMPs in combination with 1D-GE followed by high sensitivity nano-LC and MS analysis.

Statistics

In the first two papers Mann-Whitney was used for statistical analysis. However, we used Student's t-test for the last two papers because this test could be more appropriate for our comparisons between groups. The analysis was made using Prism software system (GraphPad Software Inc, San Diego, California). $P<0.05$ was considered significant. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$, ns; not significant.

RESULTS AND COMMENTS

Induction of CXC and CC chemokines in the gastric mucosa and protection against experimental *H. pylori* infection after oral administration of CpG ODN (Paper I)

The impact of oral administration of the Th1 tilted immunostimulator (79, 82) CpG ODN on induction of immune responses against experimental *H. pylori* infection was studied in mice. This included studies of the induction of innate immune responses, e.g. chemokine production, in the stomach, small intestine, MLN and serum at various time points after oral administration of CpG ODN to naïve mice. In addition, we also sought to evaluate the impact of CpG ODN administration on an established *H. pylori* infection.

Chemokine production after oral CpG ODN administration

The chemokines that were studied are known to be important for recruitment of various immune cells (Table 4). Furthermore, RANTES, together with MIP-1α and MIP-1β are associated with a Th1 type of immunity, and IP-10 preferentially recruits T cells.

Table 4. Chemokines studied in Paper I.

Chemokine	Produced by	Recruitment of
IP-10 (CXC)	Monocytes, lymphocytes, endothelial cells	T cells (120)
RANTES (CC)	Monocytes, macrophages, T cells, epithelial cells, eosinophils	Monocytes, T cells, NK cells, DC (121)
MIP-1α (CC)	Monocytes, macrophages, lymphocytes, neutrophils, eosinophils	Monocytes, T cells, neutrophils, NK-cells, DC (120)
MIP-1β (CC)	Macrophages, T cells, B cells, neutrophils,	Monocytes, T cells, basophils (120)

We found that administration of a single dose of murine specific CpG ODN to naïve mice resulted in a biphasic secretion of all the chemokines studied, as shown by analysis of saponin extracts of stomach and small intestine. The increased production of these chemokines could neither be detected in MLN, nor in the blood after oral administration, suggesting that the immune responses induced were specific to the gastrointestinal tract and not systemic. In addition, increased production of IP-10 and RANTES could be detected both in the stomach and small intestine, whereas MIP-1α

could only be detected in the stomach and MIP-1β in the small intestine. A few hours post CpG ODN administration, the first peak of chemokine production was seen in the small intestine and stomach; peak responses of RANTES, MIP-1α and MIP-1β were seen before that of IP-10 (Fig. 6 and paper I). This may suggest that the increased production of the CC chemokines (RANTES, MIP-1α and MIP-1β) is a prerequisite for induction of an increased expression of IP-10; i.e. CC chemokines recruit cells that produce IP-10 or stimulates cells for such production. In addition, second peaks were also observed that lasted for up to, or more than one week (last time point examined) post CpG ODN administration. This suggests that the source of the early chemokine response (hours after CpG ODN administration) is tissue resident immune cells and the second peak of chemokine response (days after CpG ODN administration) is from cells specifically recruited to the tissue. Indeed, staining for macrophages confirmed the presence of these cells in the gastric mucosa of naïve and *H. pylori* infected mice (data not shown).

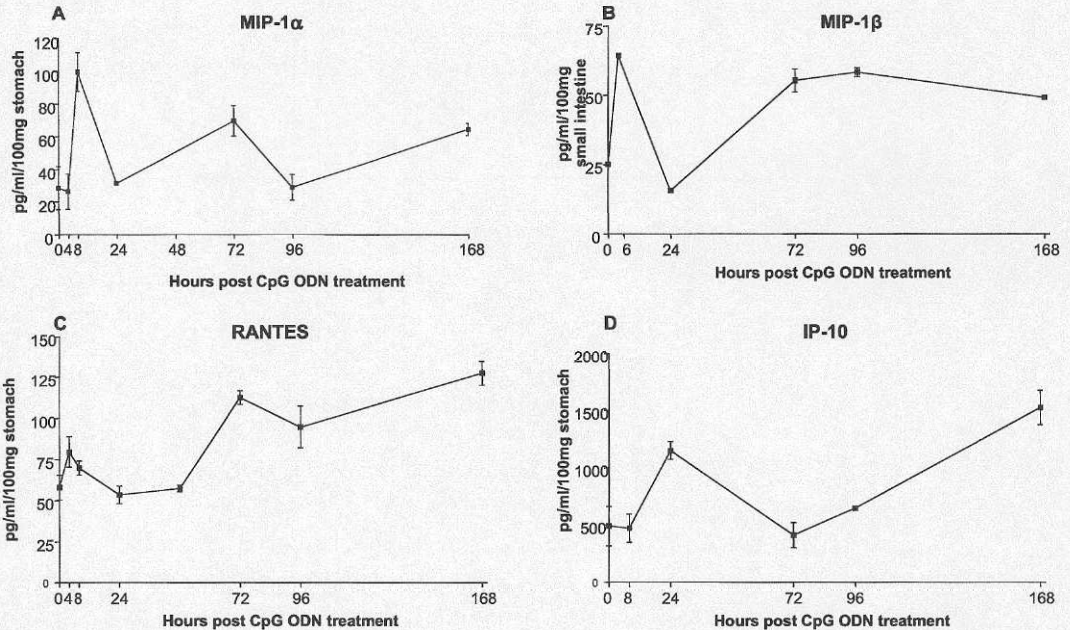


Fig. 6. Chemokine production at various time points in the gastrointestinal tract after oral CpG ODN administration.

Based on these results, the following model for induction of chemokine responses after CpG ODN administration is suggested (Fig. 7):

(1) CpG ODN binds to TLR-9 in epithelial cells and tissue resident macrophages or DC in the gastrointestinal tract. (2) Epithelial cells produce RANTES, which activates monocytes and NK cells present in the tissue and recruits new cells to the gastric mucosa. (3) Alternatively, CpG ODN stimulates DC or macrophages which secrete MIP-1α and/or MIP-1β, leading to recruitment of NK cells, monocytes and T cells. (4) Activated NK cells and T cells present in the tissue are a source of IFN-γ that can in turn (5) stimulate monocytes to produce IP-10. In addition, RANTES and IP-10 are known to recruit T cells and other cells to the GI-tract, leading to the amplification of the immune response, which could explain the second peak of chemokine secretion (6).

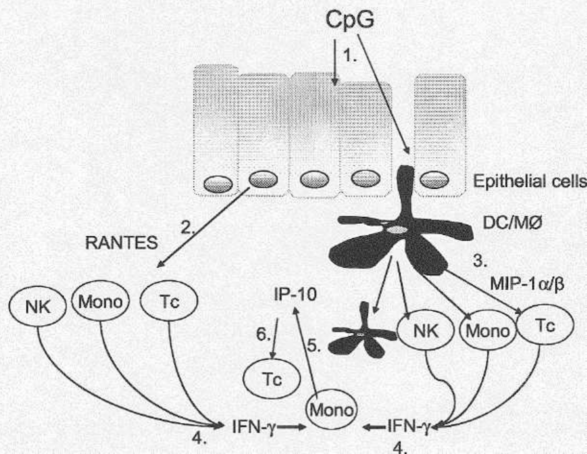


Fig. 7. A model for the induction of chemokine responses in the stomach after oral CpG ODN administration.
Mono; monocytes, MØ; macrophages, Tc; T cells

Bacterial colonization of *H. pylori* in the stomach

Because of the induction of innate immune responses after administration of CpG ODN to naïve mice, we next wanted to investigate if oral delivery of CpG ODN could induce immune responses that could reduce the bacterial load in mice with established *H. pylori* infection. To this end, *H. pylori* infected mice were immunized twice with CpG ODN and the bacterial load in infected and immunized mice were determined three days after each dose. Significant reduction in bacterial load could be detected after the second dose, but not after the first dose (I).

H. pylori infection in mice results in chronic gastritis which develops six to eight months post inoculation (104). This is similar to the findings in human chronic gastritis which is also Th1 driven, and mononuclear cells (e.g. B and T cells) as well as polymorphonuclear cells (e.g. granulocytes and macrophages) are found at the inflammatory site (122, 123). Importantly, immunization of mice quickly results in post-immunization gastritis, which resolves over time when the colonization is reduced (51, 97, 124). Indeed, the reduction in bacterial load after CpG ODN administration was reflected by an increase in infiltrating cells to the site of infection as well as an increased RANTES production locally in the stomach compared to control infected mice (I).

This study was the first to demonstrate that CpG ODN could induce mucosal immune responses and reduce the *H. pylori* load in infected mice. There have been subsequent studies showing the efficacy of CpG ODN as a mucosal adjuvant for an *H. pylori* vaccine in mice (92, 93).

Immunization with *H. pylori* lysate and CpG ODN or CTB-CpG

In an attempt to refine and improve the immunization regimen to induce further reduction of *H. pylori* colonization in infected mice, CpG ODN was conjugated to CTB and given together with *H. pylori* antigen intranasally (Nystrom J et al, unpublished). The rationale for using CTB-CpG as an adjuvant in this study was to combine the immunostimulatory effects of CpG ODN with the ability of CTB to bind to cells, especially APCs, and to shuttle CpG ODN more effectively into TLR-9 in sub-cellular compartments. Indeed, previous studies in our department have elegantly shown that the immunostimulatory effect of CpG ODN could be readily enhanced by conjugation to CTB (125) and that such conjugate confers protection against a subsequent challenge with a lethal dose of herpes simplex virus type 2 (Adamsson J, unpublished data). We found that intranasal immunization of mice with *H. pylori* lysate in combination with CpG ODN or CTB-CpG induced significant protection against a subsequent *H. pylori* challenge, but conjugation of CpG to CTB did not improve the adjuvanticity of CpG against *H. pylori* infection (Fig. 8). However, intranasal immunization with CpG ODN and lysate required less adjuvant than oral administration (I) (5 and 30 μg, respectively) and also lasted longer than oral CpG ODN administration.

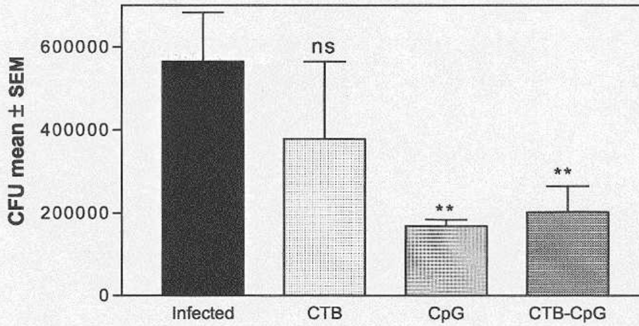


Fig. 8. Protection against challenge with *H. pylori* by intranasal immunization with *H. pylori* lysate together with CTB, CpG or CTB-CpG.

Groups of mice (n = 15 to 20) were immunized intranasally with *H. pylori* lysate together with CTB, CpG ODN or CTB-CpG 3 times, 10 days apart. Two weeks after the last immunization, the mice were challenged intragastrically with *H. pylori*. The mice were sacrificed two weeks after challenge, and the bacterial loads were quantified by culture. ns; not significant, **; $p<0.01$ compared to control infected mice. Results shown are pooled from two independent experiments.

Induction of protection by different routes of therapeutic immunization and relation between protection and mucosal immune responses against *H. pylori* (Paper II).

For the design of an effective candidate vaccine against *H. pylori* infection, immune responses that may predict the outcome of an immunization regimen (sterilizing protection or reduction in bacterial load in the stomach) should be identified, particularly after therapeutic immunization. In this study we compared the bacterial colonization in the stomach of *H. pylori* infected mice after oral and systemic therapeutic immunization, respectively, using immunization strategies (*H. pylori* lysate + adjuvant, Fig. 3) that have previously been shown to be effective for prophylactic immunization (88, 91). We also evaluated whether systemic and/or mucosal immune responses may be used as markers to predict protection against an ongoing *H. pylori* infection.

Bacterial colonization of *H. pylori* in the stomach after therapeutic immunization
We found that oral immunization of infected mice with *H. pylori* lysate and CT, given four times at weekly intervals, induced a significant reduction in bacterial load compared to in control infected mice ($p<0.01$) (Table 5 & Paper II). In contrast to the oral immunization, two ip immunizations given with a two week interval failed to induce protection against *H. pylori*. To exclude the possibility that the different outcomes of the two immunization regimens were due to the different number of immunizations, we also studied colonization in mice orally immunized twice with a two week interval. Interestingly, two oral immunizations every other week were even more effective than four weekly doses in reducing the bacterial colonization. These results are in agreement with a previous study using prophylactic immunization, and may be due to the boosting immunization being more effective if the stimulation induced by the priming dose has faded away (52). However, there were no significant differences between immune responses induced by two or four oral immunizations (Table 5).

Systemic and local antibody responses
Both oral and ip immunization resulted in increased serum IgG+IgM titers compared to titers in control infected mice (Table 5 & Paper II). Further analysis of the specific antibodies of IgG subclasses IgG1 (Th2) and IgG2c (Th1) showed that both immunizations induced significantly higher titers of IgG1 than of IgG2c in serum ($p<0.01$) (Paper II). Thus, both immunizations induced systemic Th2 responses as judged by IgG subclasses. In contrast to the serum antibody responses, only oral immunization resulted in significantly increased IgA antibodies against *H. pylori* in the stomach (Table 5 & Paper II). Hence, in spite of the strong serum antibody response, therapeutic ip immunization did not induce local IgA responses against *H. pylori*. However, although *H. pylori*-specific IgA antibodies can be regarded as an

indicator that the immune response has reached the mucosal surface, we could not demonstrate a direct correlation between specific IgA levels and the bacterial load in individual mice. Other studies have shown increased levels of *H. pylori*-specific mucosal IgA in fecal extracts after oral immunization with *H. pylori* antigen and a strong mucosal adjuvant (116, 117, 126). The present study supports and extends these findings, since the saponin-extraction method allows determination of where in the gastrointestinal tract the antibodies are produced and secreted. Thus, we could show that oral immunization gives rise to induction of *H. pylori*-specific IgA antibodies in the stomach.

Cellular immune responses

To be able to study the mucosal T cell response in infected and immunized mice, we first had to develop a new method for determination of *H. pylori*-specific $CD4^{+}$ T cell responses (II). Ideally we should evaluate the $CD4^{+}$ T cell responses directly in the stomach or in the gastric lymph nodes but this precludes assessment of protection and T cell response in the same mice. One alternative possibility, which has been used by other groups after prophylactic immunization (91, 97, 119), is to investigate the cellular immune responses in the spleen against *H. pylori* antigens. However, we could show that the splenic cellular immune response did not reflect mucosal immunity, since the oral therapeutic immunization which induced protection against *H. pylori*, was not able to induce any antigen-specific proliferation of splenic cells (Fig. 4). Instead, we evaluated if MLN could be used as a predictor of gastric immunity and a source for mucosal $CD4^{+}$ T cells. Antigen stimulation of the total MLN cell population seemed to reflect mucosal immunity because orally immunized mice (which were protected) showed higher proliferation than control infected and ip immunized mice (which were not protected). Since $CD4^{+}$ T cell responses have been shown to be important for protection against *H. pylori* (91, 95-97), we further wanted to investigate the antigen-specific response of $CD4^{+}$ T cells from MLN. However, culturing purified $CD4^{+}$ T cells with B cells or DC as APC together with *H. pylori* lysate resulted in death of the stimulated cells. This may be due to the VacA which is present in the lysate and has the ability to block T cell activation (42). To circumvent this toxicity, the APC were first pulsed with antigen over night, thereafter any unprocessed antigens were washed off and the APC were cultured with $CD4^{+}$ T cells (Fig. 5).

Similarly to the IgA response, only the oral immunization induced substantially increased antigen-specific $CD4^{+}$ T cell responses, whereas such increase was not detected after ip immunization. This suggests that protection against *H. pylori* was related to effective mucosal $CD4^{+}$ T cell responses (Table 5 & Paper II). In addition, analysis of the cytokine production showed that cytokines were mainly detected in cell cultures from orally immunized mice and the production was related to the degree of proliferation of the cells. The cytokine profile of the $CD4^{+}$ T cell responses was of the Th1 type (II), because there was a substantially increased production of IL-2, TNF-α and IFN-γ whereas no IL-4 or IL-5 were found in the supernatants. The low

Th2 response seen could be due to that IL-4 or IL-5 were rapidly utilized by cells, making them undetectable in the supernatants. We however ruled out the production of Th2 cytokines by the T cells at the mRNA level in the cells isolated from the different groups of mice and found no expression for the IL-4 gene. Thus, protection against an ongoing *H. pylori* infection was reflected by increased mucosal $CD4^+$ T cell responses with a Th1 profile.

Table 5. Bacterial load and immune responses after oral and ip immunization.

	Oral immunization		**Ip**	**Infection**
	2 doses	**4 doses**	**immunization**	**controls**
Fold decreases in CFU[a]	25***	10 ***	2.5	
$CD4^+$ T cell proliferation[b]	19 ± 11**[c]		2.2 ± 1.2	3.3 ± 1.8
Mucosal IgA[d]	130 ± 47 **	171 ± 53 **	5 ± 2	5 ± 1
Serum IgG+M[d]	5416±3367***	13013±4484***	64216 ±9234***	711 ± 423

[a] Fold decrease in colonization in the stomach was calculated as the ratio between geometric means (GM) of CFU in immunized and control infected mice.
[b] Data shown as stimulation index (SI). SI was determined by calculating the ratio between the proliferation of $CD4^+$ T cells stimulated with antigen or medium alone.
[c] Data shown is combined from two and four oral immunizations.
[d] Titers expressed as GM ± SD
**; p<0.01, ***, p<0.001 compared to control infected mice.

Because administration of CpG ODN induced high chemokine production in the stomach and small intestine (Paper I), we wanted to investigate whether oral immunization with *H. pylori* lysate and CT could induce such production as well. Indeed, orally immunized mice had an increased production of RANTES and IP-10 in their stomachs compared to control infected mice (data not shown). In contrast to CpG ODN administration, immunization with CT alone failed to induce such production. However, in this study, the chemokine production was evaluated two weeks after the last booster at variance with paper I where the chemokine response was determined up to one week post CpG administration. Furthermore, we have not studied post-immunization gastritis after immunization in this study because a previous report by our group has shown that oral therapeutic immunization with lysate and CT induces post-immunization gastritis (89) and others have shown that therapeutic ip immunization with lysate and alum did not induce significant gastritis (127).

In summary, in this study we have shown that the oral, but not the ip, immunization protocol used was capable of inducing protection against *H. pylori*. The protection was related to increased *H. pylori*-specific responses; both mucosal IgA levels in the stomach as well as Th1 biased $CD4^+$ T cell responses.

HpaA as a candidate antigen for a therapeutic vaccine against *H. pylori* (Paper III & IV)

The optimal candidate antigen in a bacterial vaccine should be surface exposed, species-specific, conserved (i.e. present in all strains) and immunogenic (i.e. capable of stimulating the immune system). HpaA fulfils all these criteria; it is present on the bacterial surface (22, 23) and is expressed by all *H. pylori* isolates tested (26). In addition, HpaA is immunogenic in humans (25, 128). Furthermore, HpaA does not have any homologies or similarities with any other known proteins (27, 28). Taken together, these data suggested to us that HpaA may be important for *H. pylori* virulence and a possible candidate antigen for use in an *H. pylori* vaccine.

Therefore, to investigate the importance of HpaA for *H. pylori* infection in mice, we introduced a previously described mutation (19) of *hpaA* into the mouse adapted strain SS1 by natural transformation. The insert in the isogenic mutant was confirmed by PCR and immunoblotting. However, in order to verify that the mutation had not caused any damage on downstream genes, i.e. *omp18* (HP0796) and *tig* (HP0795), that are being cotranscribed with HpaA (Fig. 9) (22) or second site mutations e.g. changes in other parts of the genome, we used RT-PCR and proteomic methods to characterize the mutant (III). The proteomic approach was chosen because it enables monitoring of changes in protein expression without prior knowledge of what those changes might be, as in the case of second site mutations. In addition, this approach has been used in other studies for characterization of bacterial mutants (129, 130).

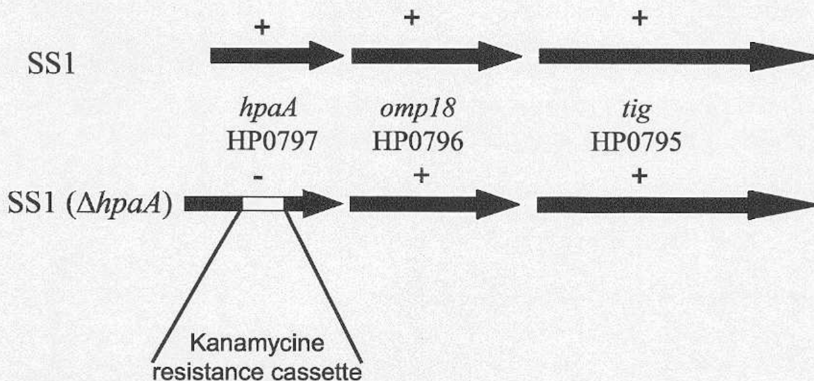


Fig. 9. Localization of *hpaA* and its downstream genes *omp18* and *tig*.
hpaA was functional in the wild-type (SS1) but not in its isogenic mutant. Omp18 and Tig could be detected in similar amounts in the wild-type and mutant strains by RT-PCR and DIGE-analysis, respectively.

Comparison of the major proteome components of SS1 and SS1 (Δ*hpaA*)

To examine the effects on the overall protein profile of SS1 and its isogenic mutant, we performed DIGE analysis on whole cell lysates of the two strains (III). Over 800 protein spots could be detected, and of these 13 spots corresponding to 11 proteins showed small changes in expression levels in the mutant compared to the wild-type strain ($p<0.05$).

Two of the down-regulated proteins, the gamma-glutamyl transferase (GGT) and the non-heme ferritin protein (Pfr) have previously been pointed out as potential virulence factors important for *H. pylori* colonization. A recent study showed that GGT provides some advantage in colonization, but it was not essential for establishment of *H. pylori* infection (131). Pfr, which is a major iron storage protein of *H. pylori* (132-134) was previously shown to have an essential role for the iron resistance of *H. pylori* (133). In addition, a pfr mutant was unable to colonize gerbils (135). Moreover, while the mutant could grow as well as the wild-type under standard conditions, its growth was significantly decreased when grown in a medium with acidic pH supplemented with ferrous iron. The HpaA mutant showed no such changes in growth rate under the same conditions as described above (data not shown). Furthermore, it has previously been shown that minor changes normally occur within a bacterial strain (136, Carlsohn E; unpublished data). Taken together, these minor variations in protein expression between the wild-type and its isogenic mutant should not affect the colonization ability of the HpaA mutant.

The most important finding of the DIGE analysis was that the Tig protein could be detected in similar amounts in both strains (Paper III). Furthermore, all the OMPs that were detected in the wild-type strain, apart from HpaA, were also expressed in the mutant strain (III). The Omp18 protein could not be detected in either strain but to the best of our knowledge, it has never been detected which may indicate that it is not being translated. Nevertheless, we could detect Omp18 mRNA transcripts both in SS1 and its isogenic mutant by RT-PCR, suggesting that the mutation had not caused any damage on the *omp18* gene (data not shown). In vitro of the wild-type and mutant SS1 strains showed that both strains grew equally well in liquid shaking culture, as measured by absorbance at OD_{600} and viability (Table 6 and Paper III).

Detection of bacteria in *H. pylori* infected mice

Because both proteomic and in vitro culture studies of SS1 and its isogenic mutant SS1 (Δ*hpaA*) showed high similarity between the two strains we next moved on to evaluate the role of HpaA for *H. pylori* colonization. This was done by infecting mice with either strain and determine the colonization at various time points post-inoculation by quantitative culture and *H. pylori*-specific PCR. While infection of mice with SS1 resulted in heavily colonized mice at all time points examined (3 days, 3 and 8 weeks), inoculation with the mutant strain failed to establish *H. pylori* colonization at any time point (Table 6). Furthermore, complementation experiments

to show that reintroduction of HpaA expression to the mutant restores its ability to colonize mice have been undertaken.

We also analysed the serum antibody response in the infected mice against both *H. pylori* MP and HpaA by ELISA. As expected, mice infected with the wild-type, but not the mutant strain, showed high IgG+IgM antibody levels against both antigen preparations (Table 6). Detection of antibodies against HpaA in mice infected with SS1 supports the immunogenicity of this antigen.

Thus, we could show that HpaA is an *H. pylori*-specific colonization factor in mice, establishing for the first time a physiological role of HpaA in vivo.

Table 6. Properties of the wild-type and HpaA-negative mutant strain

	SS1	**SS1 (Δ*hpaA*)**
Growth in vitro	+++	+++
Viability in vitro	+++	+++
Number of CFU in infected mice	6.5×10^6	Not detectable (<200)
Detection of UreA in infected stomachs by PCR	+++	Not detectable
Serum IgG+M against MP[a]	3550 ± 389**	269 ± 78[ns]
Serum IgG+M against HpaA[a]	559 ± 262**	88 ± 53[ns]

[a] Titers expressed as GM ± SD (n=5).

ns; not significant **; p<0.01, compared to uninfected mice.

Identification of protective antigens

We next wanted to identify candidate antigens for a therapeutic vaccine against *H. pylori*. Because we could show in paper II that oral immunization with *H. pylori* lysate could induce protection against *H. pylori* and that it was related to antigen-specific mucosal T cell responses, we sought to identify antigens in the lysate that may contribute to the protective immune responses. To this end, MLN cells from control infected as well as lysate immunized mice were stimulated with antigens that are known virulence factors and present in the lysate i.e. urease, Nap, Cag, catalase, and HpaA. While urease, Nap and Cag induced significantly stronger proliferative responses in immunized mice than in control infected mice (IV), catalase and HpaA did not induce any such increased response. The proliferation of urease and Nap-stimulated cells correlated to the bacterial load seen in individual mice ($p<0.01$ and $p<0.05$ respectively, data not shown).

Protection against *H. pylori* infection after immunization with HpaA

The fact that immunization with lysate did not induce any HpaA-specific cellular response was somewhat surprising; especially in the light of HpaA being a colonization factor and that immunity against HpaA may therefore be important for protection against *H. pylori*. However, the low immune response could be due to HpaA being present in relatively low amounts in the lysate. To evaluate if immune responses against HpaA may protect against *H. pylori*, mice were immunized orally with HpaA and CT. Indeed, HpaA was shown to be a protective antigen against *H. pylori* in mice and the protection was even more effective when mice were immunized orally with HpaA together with urease (Table 7 and paper IV). In fact, in some mice sterilizing immunity had been induced (i.e. no culturable bacteria were detected) after oral immunization with HpaA alone or together with urease. However, ip immunization with HpaA and Alum did not induce any significant reduction in bacterial load.

When comparing the reduction in bacterial load after immunization with the different antigen preparations used in this thesis, immunization with HpaA in combination with urease induced the greatest reduction in bacterial load (Table 7). Thus, based on these results it may be suggested that a therapeutic vaccine against *H. pylori* should optimally contain a cocktail of a few antigens, including HpaA and urease.

Table 7. Comparison of reduction of bacterial load after different immunization regimens (Paper II, IV).

Vaccine	Route	GM bacterial load ($log_{10} \pm SD$)	Fold decrease[a]
Control infected mice		6.3 ± 0.23	
Lysate + CT	Oral	4.9 ± 0.6 (2 doses)	25***
		5.3 ± 0.8 (4 doses)	10***
Lysate + Alum	Ip	5.9 ± 0.8	2.5
HpaA + CT	Oral	4.1 ± 1.5	160***
HpaA + urease + CT	Oral	3.5 ± 1.2	630***
HpaA + Alum	Ip	5.7 ± 0.2	4

[a] Compared to control infected mice. ***, $p<0.001$.

Immune responses against HpaA after therapeutic immunization

Similarly to the immune responses induced by *H. pylori* lysate immunization, protected mice exhibited increased antigen-specific $CD4^{+}$ T cell proliferation of the Th1 type and also increased titers in HpaA specific mucosal IgA antibodies compared to control infected mice (IV). Because sterilizing immunity against *H. pylori* had been induced in some mice it would be of interest to look into individual immune responses, particularly in these mice. However, there was no relation

between mucosal IgA responses and protection in individual mice, and the $CD4^+$ T cell analysis was performed on cells that were pooled from different mice in order to get enough cells, but it seems plausible that the difference in protection could be explained by the degree of T cell responses in individual mice.
The antibody response against HpaA was relatively low in control infected mice suggesting that HpaA is a weak immunogen during infection. The same holds true in humans, where *H. pylori* infected individuals have low mucosal antibody responses against HpaA, e.g. low levels of HpaA-specific SIgA and antibody secreting cells were detected in the stomachs of asymptomatic individuals and duodenal ulcer patients (25, 137). Our studies in mice show that it is possible to boost a strong immune response against HpaA in mice by therapeutic immunization (IV). However, our preliminary data suggest that priming, e.g. by infecting mice may be a prerequisite for mounting high immune responses because HpaA immunization of mice, in the absence of infection, failed to induce proliferative responses in MLN (data not shown).
In summary, these studies clearly show that HpaA is a *H. pylori*-specific colonization factor and a promising candidate antigen for a therapeutic vaccine against *H. pylori*.

GENERAL DISCUSSION

H. pylori infects more than half of the world's population, making it one of the most common gastrointestinal infections world-wide. The current antibiotics-based treatment against *H. pylori* is associated with several drawbacks e.g. high cost, low compliance and most importantly the rapid increase of antibiotics resistance. A vaccine against *H. pylori* would therefore be a cost-effective and attractive alternative or complement to the current treatment.

A vaccine can be given either prophylactically; i.e. before an individual becomes infected, or therapeutically; i.e. against an already established infection. A therapeutic vaccine would also have the advantage over the antibiotics-based therapy in that it provides appropriate immunological memory to avoid re-infections (40, 51, 89). Experimental vaccine studies in mice have shown that both prophylactic and therapeutic immunizations have conferred protection against *H. pylori* (40, 88-90). Most immunization studies against *H. pylori* infection in mice have used prophylactic regimens. However, the focus of this thesis has been on therapeutic immunization. One reason for this is earlier studies in our group showing that gastric immune responses could not be induced in uninfected, but only in *H. pylori* infected individuals, when given a model vaccine (138). We have also focused on using a mucosal immunization strategy against *H. pylori* to induce immunity at the site of infection. In addition, other advantages of mucosal immunization includes the ease of administration and the reduced, or minimal, risk of e.g. HIV and Hepatitis B virus transmission that can occur with injectable vaccines.

In this thesis a well established mouse model of *H. pylori* infection (51, 52, 104) has been used to study the immune responses and protection induced by therapeutic immunization. However, it is important to keep in mind that results from mouse studies may not be translated directly into humans. This is especially true for studies using knock-out mice in order to elucidate components of the immune system that may be important for protection against *H. pylori*. On the other hand, recent reports support the validity of using wild-type mouse models of *H. pylori* for vaccination studies. First, *H. pylori* proteins that are expressed in infected mice and are exposed to the mouse immune system appear to be similar to those in human *H. pylori* infections, suggesting that *H. pylori* models may be suitable for screening antigens for human vaccination (113). In addition, a human vaccination and challenge model has recently been established (139, 140). In this model, human volunteers were vaccinated with live typhoid vaccine (Ty21a) expressing urease before challenge with an antibiotics-susceptible *H. pylori* strain lacking CagPAI and other antigens associated with severe disease outcome. Although only a few of the vaccinees were cleared of their infection, analysis of the immune response induced in these

individuals revealed that, similar to protective immune responses in mice (II, 95-97, 141), CD4$^+$ T cell response of the Th1 type was associated with protection. These findings support that the mouse model may be used for identification of protective immune mechanisms and candidate antigens.

Many immunization studies against *H. pylori* infection in mice have used powerful mucosal adjuvants (51, 88-90), e.g. CT and LT, which cannot be used in humans because of their toxicity (65). Therefore there is a great need to develop a safe and non-toxic adjuvant for mucosal use.
Bacterial DNA has been shown to stimulate the vertebrate innate immune system, and it is CpG dinucleotides with appropriate flanking regions that are responsible for this activation through binding to TLR-9 (80, 81). Because of its immunostimulatory effect, CpG ODN has been evaluated as an adjuvant both in mice and in humans (84, 85, 142). When we started this study, systemic administration of CpG ODN had been shown to confer protection against different infections in mice such as *Leishmania major* (142) and *Listeria monocytogenes* (143). In addition, studies in our laboratory had demonstrated that vaginal-mucosal administration of CpG ODN induced protection against genital herpes infection and disease (144). However, the adjuvant effect of CpG ODN on the gastric mucosa had not been studied. We therefore sought to investigate whether oral CpG ODN administration could influence the innate immune responses in the gastrointestinal mucosa and also to evaluate the impact of CpG ODN on an established *H. pylori* infection (I). CpG ODN is an interesting adjuvant against *H. pylori* infection as it induces Th1 polarized responses and recently several studies (97, 145) have shown that Th1 and not Th2 responses (146) are necessary for protection against *H. pylori*. Indeed, we could show that a single dose of CpG ODN could induce a rapid and strong induction of the chemokines RANTES, MIP-1α, MIP-1β and IP-10 in the gastrointestinal mucosa (I). However, no increase of these chemokines in the mesenteric lymph nodes or sera of these mice could be detected, suggesting that CpG ODN has a local effect. In addition, administration of CpG ODN to *H. pylori* infected mice resulted in a significant decrease in the bacterial load, which was associated with infiltration of immune cells to the gastric mucosa and an increased RANTES production.

There have been concerns that CpG ODN could enhance the immunogenicity of self-antigens, which could lead to development of autoimmune diseases. However, repeated ip immunizations of high doses of CpG ODN did not cause any adverse effects in mice (147). In addition, clinical studies using CpG ODN as part of a vaccine strategy against hepatitis B or cancer have reported that CpG ODN is well tolerated by the patients (84, 85). Thus, CpG ODN seems to be a safe adjuvant that can be administered mucosally. However, the optimal dose of CpG ODN required for administration to the mucosal site in order to achieve protection against *H. pylori* remains to be evaluated.

We next wanted to compare protection induced by different routes of immunization and also to identify immune responses that could reflect protection induced by therapeutic immunization, i.e. find markers for protective immunity. To this end, mice were orally or ip immunized with *H. pylori* lysate and adjuvants using protocols that have conferred protection when used prophylactically (88, 91). In contrast to the prophylactic immunization, only the oral, but not ip immunization with *H. pylori* lysate and adjuvant was able to induce protection against *H. pylori* when given therapeutically (II). Furthermore, we could clearly demonstrate that oral, but not ip, therapeutic immunization resulted in significantly increased antigen-specific $CD4^+$ T cell responses in MLN (II). Analysis of the cytokine production showed that cytokines were mainly found in T cell cultures from orally immunized mice, and that they had a Th1 profile; i.e. they produced increased levels of IFN-γ and TNF-α but no IL-4 or IL-5. This is in line with previous studies showing the importance of Th1 responses for protection against *H. pylori* (97, 145). Our study is the first to make a comprehensive comparison on the effect of two different immunization routes on the mucosal T cell responses and protection against *H. pylori* infection in immunocompetent mice.

The role of antibodies, in particular mucosal IgA, for protection against *H. pylori* is less clear than the role of T cells. Knock-out studies have shown that B cells and antibodies are dispensable for protection against *H. pylori* (95, 100). However, preincubation of *H. felis* with monoclonal IgA antibodies against urease protected mice from infection (99). In addition, patients with IgA deficiency have been shown to have increased risk of developing gastrointestinal carcinomas (102, 103) and gastric adenocarcinoma patients have decreased production of gastric IgA antibodies (Quiding-Järbrink et al., submitted for publication). These studies show that a protective, albeit not necessarily essential, role for antibodies against *H. pylori* infection and *H. pylori*-associated diseases cannot be ruled out. Evaluation of *H. pylori*-specific mucosal IgA antibodies in the stomach of infected and immunized mice showed that only orally immunized mice had significantly increased levels of IgA antibodies compared to control infected mice (II).

The ip immunization regimen with alum as adjuvant, which induces protection when used prophylactically (91) did not result in decreased *H. pylori* colonization after therapeutic immunization (II). It does not seem to be the parenteral immunization *per se* that only works prophylactically, because Sanchez et al. have shown that subcutaneous therapeutic immunization with antigens together with the cationic liposome DC –Chol as adjuvant can induce protection against *H. pylori* (148). However, T cells seemed to be important for the protection induced by alum because transfer of $CD4^+$ T cells from immunized mice to otherwise immunodeficient *rag*$1^{-/-}$ recipients induced protection against *H. pylori* infection in these mice (91). Furthermore, we could show that prophylactic, but not therapeutic ip immunization, induced mucosal T cell responses (II) and this discrepancy could explain the

differences in protection after prophylactic and therapeutic ip immunization. One possible reason for the lack of protection against *H. pylori* after therapeutic ip immunization with alum as adjuvant could be the induction of functional *H. pylori*-specific $CD4^+CD25^+$ regulatory T cells (Treg) in the gastric mucosa (149, 150), which could have immunosuppressive effects on the immune responses against the *H. pylori* infection. Thus, it is tempting to speculate that expansion of Treg during an ongoing *H. pylori* infection suppresses the induction of immune responses that only oral therapeutic immunization with a strong mucosal adjuvant such as CT can overcome whereas the ip immunization with alum cannot. This suggests that different magnitudes of immune responses or possibly different immune mechanisms are required to provide protection against *H. pylori* infection after therapeutic compared to prophylactic immunization.

The mechanisms for the protection induced by therapeutic immunization in our mouse model of *H. pylori* infection are still not fully understood. One possible model for how protection is induced against *H. pylori* following immunization and mediated by the immune system may be as follows:
The immunostimulatory effect of CT used as adjuvant for oral immunization enhances uptake of the coadministered antigen in the intestine and stimulates antigen presentation (58, 60). The activated B and T cells will thereafter home to the gastric mucosa. Thus, the oral immunization with antigen and CT leads to increased antigen-specific B and T cell responses against the antigen in the stomach. Plasma cells will secrete *H. pylori*-specific IgA antibodies into the gastric lumen where they adhere to the mucus and prevent the bacterium from binding to the epithelial cells. The activated T cells, predominantly of the Th1 type in the stomach, can secrete IFN-γ to activate tissue resident and recruited macrophages. The effector T cells or the activated macrophages could in turn influence the epithelial cells by cytokine secretion. Thus, the epithelial cells may be the "ultimate effector cell" and function as a bridge between the $CD4^+$ T cells and *H. pylori* by affecting the bacterial colonization in a number of ways:

- ◊ Secretion of defensins. Indeed, it has been shown that α-defensins (that act by damaging the bacterial cell wall) and factors involved in epithelial maintenance/defence are upregulated in *H. pylori* immunized and protected mice (151).
- ◊ Increased epithelial cell turnover (151). This may be disadvantageous for the colonization of *H. pylori* because the bacteria adhere to epithelial cells.
- ◊ Alteration and/or increase of the mucin secretion (151, 152). For example, changes in the viscosity of the mucus may impair the motility of *H. pylori* (153). In addition, a human gastric mucin has been shown to have antimicrobial activity against *H. pylori* (154).

Many other cell types and signals have also been suggested to be involved in diminishing or clearing the *H. pylori* infection. Inducible nitric oxide synthase (iNOS) is upregulated in the gastric mucosa of immunized and protected mice (141). However, mice lacking iNOS are equally well protected as wild-type mice, suggesting that NO is probably not involved in protection but may contribute to the inflammation (141). In addition, adipokines, e.g. cytokine-like molecules produced by mature adipocytes and some other cell types, have recently been implied as a link between the endocrine and immune system and such molecules have been found to be upregulated in vaccinated and protected mice (151, 155). One hypothesis of their function may be that they stimulate gastric T cells or epithelial cells. All together, the combination of many of these effector molecules and events may result in suppression of *H. pylori* colonization in the stomach of immunized mice.

An important aim of these studies was to try to identify protective vaccine candidate antigens. Because HpaA has several properties of an optimal candidate antigen, e.g. it is surface located, immunogenic and present in all *H. pylori* strains but not in other species, we wanted to evaluate its contribution to *H. pylori* colonization and immunity (III). By infecting mice with the mouse adapted strain SS1 or its isogenic mutant SS1(Δ*hpaA*) we could clearly demonstrate that HpaA is essential for *H. pylori* colonization in mice because only infection with the wild-type, but not the mutant, resulted in *H. pylori* colonized mice.

However, the function of HpaA is still unknown. Although originally described to be an adhesin, our HpaA mutant displayed the same binding properties as the wild-type strain for binding both to previously identified *H. pylori* binding glycosphingolipids and to gastric cell lines (Teneberg S, unpublished data, 19). It may therefore be possible that HpaA has a role unrelated to adhesion, e.g. HpaA may facilitate colonization of *H. pylori* in the gastric mucosa. For example, *hpaA* is cotranscribed together with *omp18* and *tig*, which may suggest that their functions are related. The function of Omp18 is unknown, but based on sequence homologies Tig has been suggested to be involved in peptide secretion and trafficking (27, 28). This, together with the fact that HpaA has been found to be present in high quantities in the inner membrane may suggest that HpaA also has a shuttle function. Notably, HpaA has been shown to directly stimulate and activate human DC in vitro (128) but it is not known through which receptor this activation is initiated.

We next wanted to evaluate the capacity of HpaA as a candidate vaccine antigen for therapeutic immunization against *H. pylori* in mice (IV). Indeed, we concluded that HpaA is a very promising candidate antigen, because oral therapeutic immunization of mice with this antigen together with CT resulted in dramatic decrease in bacterial load in the stomachs of *H. pylori* infected mice compared to in control infected mice. This effect was even stronger when mice were immunized with both HpaA and

urease together with CT. To the best of our knowledge, the reduction in bacterial load seen with this combination is comparable to or higher than any other antigen(s) used for oral therapeutic immunization against *H. pylori* in mice (40, 89, 156). This would suggest that an optimal *H. pylori* vaccine should consist of a cocktail of a few *H. pylori*-specific antigens, which could induce additive or synergistic immune responses against *H. pylori* by targeting different essential components of the bacteria or different levels in the pathogenesis. However, whether this particular combination of HpaA and urease would work as efficiently for prophylactic immunization remains to be determined, since one study has shown that the optimal antigen combinations for induction of prophylactic protection are different from the therapeutic ones (148). For example, a bivalent prophylactic vaccine with urease combined with antigen e.g. AlpA or catalase, was optimal for inducing protection against a subsequent challenge. However, therapeutic immunization was most effective when using AlpA together with BabB. In contrast to our results (IV), Sanchez et al. showed that a therapeutic bivalent vaccine consisting of urease together with AlpA or BabB did not confer any stronger protection than the mice immunized with these antigens alone. On the other hand, they used another immunization regimen (subcutaneous immunization with DC Chol) and challenge strain than we did, which may account for the different results (148).

However, identification of a candidate vaccine antigen is not enough for development of a human vaccine against *H. pylori,* because we and others have shown that the type of immunization (prophylactic vs therapeutic) (II, 88, 91), adjuvant-antigen combination (I, II, III) and timing of the immunizations (II, 52) all are important parameters that may affect the induction of mucosal immune responses against *H. pylori*. Indeed, the reason why clinical vaccine trials against *H. pylori* have so far failed is probably not due to the antigen used (e.g. urease and lysate), but rather to the fact that the mucosal adjuvants have not been able to induce sufficient protective immune responses against the coadministered antigens (78, 87).

In conclusion, the results in this thesis suggest that an optimal therapeutic vaccine against *H. pylori* should be administered orally and include an effective mucosal adjuvant. The antigens in such a therapeutic vaccine should provide protection against different key pathogenic mechanisms, e.g. HpaA and urease. Our findings further suggest that the immunization should induce antigen-specific $CD4^+$ T cell responses with a Th1 profile (IFN-γ and TNF-α) and probably also locally produced specific IgA.

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