Humoral and cellular immune responses to *Helicobacter pylori* in Bangladeshi children and adults that may be related to protection

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Cover page: Pictures showing different sites in the Mirpur study area in Bangladesh.

**Dedicated
to
the memories of my father, Zahidur Rahman**

I am satisfied that when the Almighty wants me to do or not
do any particular thing, He finds a way of letting me know it.

-Abraham Lincoln

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<u>Abstract</u>
Helicobacter pylori (Hp) colonizes the human gastric and duodenal mucosa and the infection may cause peptic ulcers and gastric adenocarcinoma. Half of the world's population is infected with Hp with the highest prevalence in developing countries. Hp infection is normally acquired during childhood, but comparatively little is known about immune responses to acute infection or potential differences in responses between individuals in Hp endemic and nonendemic countries. The overall aim of this thesis was to analyze humoral and cellular immune responses to Hp in children and adults living in a country with a high prevalence of Hp infections, i.e. Bangladesh.

T- and B-cell responses to Hp were analyzed in Hp infected adults from Bangladesh and Sweden. Comparable numbers of $CD19^+$ B cells and $CD4^+$ T cells and similar levels of Hp-specific IgA antibodies were found in gastric mucosa from Bangladeshi and Swedish subjects. However, higher numbers of $CD19^+$ B cells and higher levels of specific and total IgA antibodies were found in the duodenum of the Bangladeshis, possibly due to frequent enteric infections causing recruitment of Hp-specific and unspecific lymphocytes to this site. Furthermore, Bangladeshi subjects had about two-fold lower Hp-specific IgA and IgG serum antibody titers.

To determine the incidence of Hp infection during early childhood in a high endemic area and possible associations between infection and different host and environmental factors, a birth cohort (BC) study was undertaken in Bangladeshi children from birth up to 24 months. Using diagnostic methods suitable for use in less well-equipped laboratories, i.e. stool antigen test and serology, 50-60% of the children were found to be positive for Hp at 24 months. Most children were initially infected with Hp during spring or autumn and blood group A children had increased susceptibility to the infection. Serum and stool samples collected every third month from the BC children were analyzed for development of systemic and mucosal antibody responses to acute Hp infection. Almost all children mounted specific, ≥4-fold serum IgA and stool IgA responses following infection. Serum IgG levels at birth were comparable to the maternal antibody levels and decreased during the initial 6 months, whereafter they increased in response to infection. An association between spontaneous eradication of Hp infection (in approximately 10% of the children) and increased serum antibody responses was found. Pre-existing maternal IgG and breast milk IgA antibody levels were associated with delayed onset of Hp infection.

To analyze if certain T-cell responses (Th1 and Th17) may contribute to the immune responses against Hp, peripheral blood mononuclear cells were isolated and stimulated with Hp antigens. Cells from both Bangladeshi infants and adults responded with production of both IL-17 and IFN-γ, with higher IL-17 responses in infants. These results suggest that Th17 as well as Th1 type T-cell responses may be important for initial immune responses to Hp in young children.

These studies give important information regarding acquisition of Hp during early childhood in a high endemic country and provide clues about immune responses that may be related to protection against Hp infection.

Original Papers

This thesis is based on the following papers referred to in the text by the given Roman numerals:

I. Bhuiyan TR, Qadri F, Bardhan PK, Ahmad MM, Kindlund B, Svennerholm AM, and Lundgren A: Comparison of mucosal B- and T-cell responses in *Helicobacter pylori*-infected subjects in a developing and a developed country.
FEMS Immunol Med Microbiol. 2008 Oct; 54(1): 70-9.

II. Bhuiyan TR, Qadri F, Saha A and Svennerholm AM: Infection by *Helicobacter pylori* in Bangladeshi children from birth to two years: relation to blood group, nutritional status and seasonality.
Pediatr Infect Dis J. 2009 Feb; 28(2): 79-85.

III. Bhuiyan TR, Saha A, Lundgren A, Qadri F and Svennerholm AM: Immune responses to *Helicobacter pylori* infection in Bangladeshi children during their first two years of life and relation between maternal antibodies and onset of infection.
Submitted for publication.

IV. Bhuiyan TR, Qadri F, Janzon A, Chowdhury MI, Lundin SB and Lundgren A: Th1 and Th17 responses to *Helicobacter pylori* in Bangladeshi children and adults.
In manuscript.

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ABBREVIATIONS

APC	antigen presenting cell
AS	asymptomatic
ASC	antibody secreting cells
BabA	blood group binding adhesin A
cagPAI	cytotoxin associated gene pathogenicity island
CBA	cytokine bead array
CI	continuously infected
DU	duodenal ulcer
ELISA	enzyme-linked immunosorbent assay
ETEC	enterotoxigenic *Escherichia coli*
FCM	flow cytometry
ICDDR,B	International Centre for Diarrhoeal Disease Research, Bangladesh
IFN-γ	interferon gamma
IHC	immunohistochemistry
IL	interleukin
IP-10	IFN-γ inducible protein-10
iTregs	induced regulatory T cells
LMIC	low and middle-income countries
LPL	lamina propria lymphocyte
LPS	lipopolysaccharide
MALT	mucosa associated lymphoid tissue
MIG	monokine induced by γ-interferon
MP	membrane preparation of *H. pylori*
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohemagglutinin
RI	reinfected
SabA	sialic acid binding adhesin A
SE	spontaneously eradicated
SUH	Sahlgrenska University Hospital
TGF-β	transforming growth factor-β
Th	T helper
TNF-α	tumor necrosis factor-α
Tregs	regulatory T cells
UBT	urea breath test
VacA	vacuolating cytotoxin A

INTRODUCTION

History of *Helicobacter pylori* infection

For a long time, the human stomach was considered to be a sterile organ, where no microorganisms can survive due to the acidic conditions. However, in 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 and peptic ulcers (90). Warren and Marshall were recently awarded the Nobel Prize (in 2005) for their discovery of *H. pylori* and its role in gastritis and peptic ulcer disease. Today, *H. pylori* is also recognized as an important cause of gastric cancer and mucosa associated lymphoid tissue (MALT) lymphoma (124).

Prevalence of *H. pylori*

More than 50 percent of the population worldwide is infected with *H. pylori* with a higher prevalence in developing countries and in groups with poor socio-economic and hygienic status than in developed areas (Figure 1). The infection is generally acquired during childhood (40, 94).

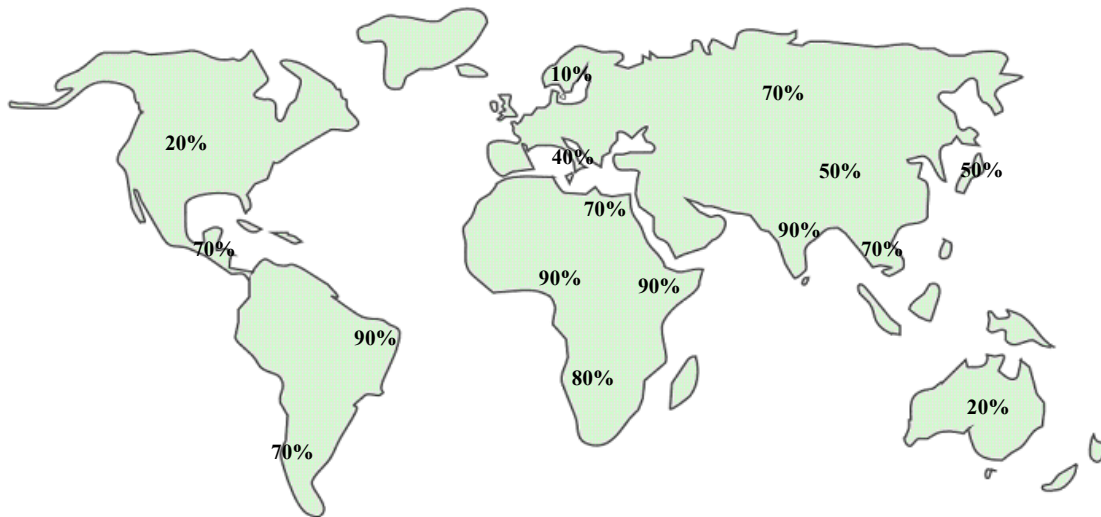**Figure 1.** Prevalence of *H. pylori* infection in adults in different parts of the world. Prevalence data cited in text or adapted from (97, 119, 152).

The prevalence of *H. pylori* in adults and children in low and middle-income countries (LMIC) and in the industrialized world varies a lot. In Africa (e.g. Ethiopia, Gambia and Nigeria), >90% of the adult populations are infected with *H. pylori* (59, 81,

139) and in Gambia, 95% of the children (<5 years) were found to be positive for *H. pylori* (139). In Latin America, e.g. Chile and Mexico, around 70% of the adults were shown to be infected with *H. pylori* and among Mexican children (<5 years), the *H. pylori* prevalence was 47% (106, 147). A high prevalence of *H. pylori* has also been observed in India both in adults (88%) (51) and children less than 5 years (57%) (100). It has previously been shown in Bangladesh that the prevalence of *H. pylori* was 42% already by 2 years of age with a rapid increase to 67% by 10 years of age (25, 117) and that as many as 84% of 6-9 years old children were *H. pylori* positive in areas where sanitary conditions were very poor (88). Very recently, the prevalence of *H. pylori* was also reported to be relatively high (50%) in Muping city in China (153). On the other hand, in industrialized countries like Sweden, only 2% of the children (10-12 years) with Scandinavian parents (140) and 11% of the adults (25-50 years) have been reported to be infected with *H. pylori* (136). Slightly higher prevalence has been observed in other Western countries such as Italy (43%) and the US (20%) (121), but it is clear that the incidence of *H. pylori* infection in developed countries is relatively low, particularly in children, with a higher prevalence in elderly people. As the socioeconomic status has improved in developed countries, the prevalence of *H. pylori* in younger generations has declined (42). The age related apparent increase in the prevalence (higher in the older generation and lower in younger generation) in developed countries could best be explained as a "birth cohort effect." However, no such effect has been noted in the developing world (42, 73).

Transmission of *H. pylori* infection

H. pylori is spread within the family and close communities (70) through the immediate environment or via environmental reservoirs or vectors. However, little is known about the main route of dissemination from an infected individual. The fecal-oral route has been suggested in most studies, although oral-oral (34, 137) or gastro-oral routes (79, 109) have been proposed by others. In addition, there are reports that *H. pylori* may be spread during episodes of vomiting and diarrhea (76). Recently, we have shown that vomitus would be the most likely source of *H. pylori* in person-to-person transmission whereas spread via contaminated water is less likely (66).

H. pylori associated diseases

Most *H. pylori* infected individuals develop chronic active gastritis. Despite this gastritis, most infected individuals remain asymptomatic. In general, the bacterium causes gastric or duodenal ulcers in approximately 10-15% of those infected and gastric cancer in another 1-2% (39). In fact, the World Health Organization (WHO) designated *H. pylori* as a class 1 carcinogen in 1994. Individuals who have severe gastric atrophy, corpus-predominant gastritis, or intestinal metaplasia have been reported to have an increased risk for development of gastric cancer. In contrast, persons who have antrum-predominant gastritis and gastric metaplasia in the duodenum have been reported to have an increased risk of developing duodenal ulcers. *H. pylori* infected persons with nonulcer dyspepsia, gastric ulcers, or gastric hyperplastic polyps may also have increased risk of developing cancer, whereas those with duodenal ulcers do not (143).

The prevalence of *H. pylori* associated diseases seems to vary considerably in different parts of the world. These differences have led to coining of the terms "the African and Indian enigmas" (52). The African enigma refers to the apparent low prevalence of peptic ulcers and gastric cancer in Africa, despite a very high prevalence of *H. pylori* infection. Similarly, the Indian enigma refers to the observation that there are areas in Asia where *H. pylori* infection is very common, such as India, Thailand, Bangladesh and Pakistan, but where gastric cancer is rare, whereas in other areas of Asia where *H. pylori* is almost equally prevalent, gastric cancer is frequent (e.g. China, Japan and Korea) (52). However, recent analyses of endoscopic data suggest that the enigmas are medical myths, as the prevalence of gastric cancer is not unusually or unexpectedly low in these parts of the world, but is rather a consequence of the antral predominant gastritis commonly found here (52). Furthermore, short life expectancies, inadequate health care systems and lack of systemic surveillance and databases are factors that are likely to affect the official statistics of *H. pylori* associated diseases in these areas of the world. The reasons for the different patterns of gastritis in different populations are however still unclear, but diet, environmental, host and microbial factors have been suggested (52). These factors are also likely to influence whether infected individuals will remain asymptomatic or develop disease, but the relative contribution of the individual factors for the risk of disease development remains to be determined.

Treatment of *H. pylori* infection

There is no single antibiotic that can be used alone for treatment of *H. pylori* infection. The current treatment consists of a combination therapy with two different antibiotics (e.g. metronidazole and amoxicillin) together with a proton-pump inhibitor, which in most cases results in successful eradication of the bacteria and healing of ulcers (150). However, there are some major drawbacks with such a 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. Furthermore, such treatment does not protect against reinfections, which frequently occur in areas with high prevalence of *H. pylori* (78, 87). In Bangladesh, the prevalence of metronidazole resistance is high (77%), which might be due to frequent use of metronidazole for other intestinal as well as gynecological problems (98). Among the *H. pylori* strains isolated in Bangladesh 15% were also tetracycline-resistant (98). In Sweden, on the other hand, resistance to these antibiotics was lower with 16% of the strains being resistant to metronidazole and 0.3% to tetracycline, most probably due to a restrictive prescription policy (130).

Prevention of *H. pylori* infection

Many therapeutic and prophylactic vaccine candidates against *H. pylori* have been evaluated in experimental animals and several of these studies have suggested that it is possible to induce protective immunity by vaccination (38, 104, 116, 134). The main candidates so far are the virulence factors of *H. pylori* discussed below, alone or in different combinations with various adjuvants (134). Whole bacterial cells and whole cell lysates of *H. pylori* and related *Helicobacters* have also been frequently tested.

A *H. pylori* vaccine should preferably work at two different levels, e.g. result in a decreased risk of developing *H. pylori*-associated disease for an individual, and to decrease the risk of infection at the population level. A prophylactic vaccine should be given before an individual becomes infected with *H. pylori*, e.g. in children, whereas a therapeutic vaccine should primarily be given to those who have already developed *H. pylori* associated disease. In addition, because chronic *H. pylori* infection, even in the absence of symptoms, is a risk factor for development of adenocarcinoma (39), vaccination of asymptomatic carriers may be justified. Furthermore, a therapeutic vaccine

would confer protection against reinfection. However, despite successes in animal models, no successful human clinical vaccine trial has been carried out so far (134).

Virulence factors of *H. pylori*

H. pylori is a gram-negative bacterium which expresses a variety of different virulence factors for survival in the stomach. *H. pylori* also colonizes areas of gastric metaplasia in the duodenum. The virulence factors include the flagellae that provides motility and different adhesion factors, e.g. blood group binding adhesin A (BabA) and sialic acid binding adhesin A (SabA), which enable *H. pylori* to adhere to surface mucosal cells and to compounds of the mucus layer and thereby avoid bacterial shedding (60, 89). Earlier findings have emphasized the importance of the adherence factors for the induction of gastric inflammation, ulcer disease, and also gastric adenocarcinoma. It has been found that bacterial colonization densities are of great importance for the degree of mucosal inflammation and damage, (10, 56, 151) and BabA appears to be a key factor favoring high colonization densities. Since presence of the *babA2* gene has previously been correlated with both ulcer disease and adenocarcinoma (46), it appears that genetic susceptibilities may influence the further development of disease once the bacterial colonization is established.

CagA is another virulence factor which seems to be associated with more severe disease outcome (17). CagA is located within the cytotoxin associated gene pathogenicity island (CagPAI, a 40kB segment containing about 30 genes) (73). Many genes in the CagPAI encode for a type IV secretion system that can deliver CagA, and possibly other proteins, into the mammalian host and thereby affect the function of the epithelial cells. Another virulence factor, VacA, induces vacuolation of epithelial cells and can block T-cell proliferation (27, 44). Both CagA[+] and VacA[+] strains are associated with more severe disease outcomes.

H. pylori also produces large amounts of the enzyme urease, which is localized inside and outside the bacterium. Urease breaks down urea (which is normally secreted into the stomach) to carbon dioxide and ammonia (which neutralizes gastric acid). The survival of *H. pylori* in the acidic stomach is dependent on urease, and the bacteria would eventually die without the enzyme (129).

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Assays for diagnosis of *H. pylori*

Various tests have been developed for the detection of *H. pylori* infection. The specific advantages and disadvantages of these techniques are summarized in table 1. Well established methods are histology and bacterial culture from gastric and duodenal biopsies, which are arguably considered "gold standards" among the different detection methods (35). However, they have relatively low sensitivities and are invasive and labour intensive. Furthermore, they require trained pathologists for histological scoring and are not suitable for diagnosis in field studies or in young children since these require collection of biopsies. In clinical settings, the Urea Breath Test (UBT) is often used as a rapid method to detect active infection. In this test, the patient ingests urea labeled with either carbon-13 or carbon-14 isotopes and the presence of the labeled isotope is measured in exhaled breath carbon dioxide. A positive test indicates that the urea has been metabolized by *H. pylori* urease. Although the UBT usually has a high sensitivity for diagnosis of infection, it is limited by being technically cumbersome, time-consuming, expensive and has low specificity in very young children (<2 years of age) (35, 69). Therefore, UBT is not an optimal diagnostic test in field settings or in young children.

Other noninvasive tests may be more suitable for such studies; e.g. stool antigen tests and/or serology. The stool antigen tests, which usually are commercial, are based on the detection of *H. pylori* antigens shed in the stool of infected subjects. Initial reports of the monoclonal antibody based enzyme-linked immunosorbent assay (ELISA) test (Amplified IDEIA™ Hp StAR™, Dakocytomation, Denmark) were very encouraging, showing 100% sensitivity and 91% specificity in adults (47).

However, measurement of serological IgG is not suitable alone for detection of *H. pylori* in children although it is relatively simple and can be an immediate option. This is because children acquire transplacentally derived IgG from their mothers, which may remain for at least 6 months. Furthermore, serology does not reflect active infection, as IgG levels remain elevated for a couple of months after eradication of the infection.

Finally, a plethora of different polymerase chain reaction (PCR) methods have been used to detect *H. pylori* DNA in clinical specimens. However, the PCR based methods are normally primarily used for research and not for diagnostic purposes.

Table 1. Diagnostic methods used for detection of *H. pylori*.

	Sensitivity	Specificity for *H. pylori*	Time required	Bacterial detection Live	Dead	Remarks
Invasive methods						
Histology	Low	High	1-2 days	+	+	Gold standard, inexpensive
Culture	Low	High	5-7 days	+	-	Alternate gold standard, inexpensive
Rapid urease test (CLO)	Low	High	<1 day	+	-	Rapid, cost-effective
Noninvasive methods						
Urea breath test	High	High	<1 day	+	-	Alternate gold standard, not appropriate for resource poor settings or young children, relatively expensive
Fecal antigen test	Medium	High	1 day	-	+	Simple, sensitivity increases with testing of multiple samples, expensive
Serology	Medium	High	1-2 days	na	na	Useful for screening and epidemiological studies, inexpensive
PCR (biopsy)	High	High	1 day	+	+	Relatively simple, inexpensive

na; not applicable

Immunology

Overview of immune responses to H. pylori

In response to *H. pylori* infection, the host mounts systemic as well as mucosal immune responses with associated gastritis shortly after onset of infection. The mucosal responses are characterized by massive infiltration of neutrophils into the gastric mucosa (128) which is followed by activation of dendritic cells and recruitment of B and T cells (53,

73, 102). During chronic infection, neutrophils remain in the mucosa together with lymphocytes, which are scattered throughout the mucosa, but may also form lymphoid follicles, consisting of a central B-cell area surrounded by a thinner layer of mainly CD4$^+$ T cells (45, 135). Follicles are present in the gastric mucosa of virtually all infected individuals, but their function is still unknown (45). *H. pylori* specific IgA antibodies are produced both locally and systemically (91, 92). A T-cell response is also induced and interferon-γ (IFN-γ) producing helper T cells seem to be an important component of the response (8, 11, 30, 84). However, recent data suggest that interleukin-17 (IL-17) producing helper T cells as well as regulatory T cells may also play important roles during infection (105).

General properties of CD4$^+$ T -cell responses

CD4$^+$ T helper (Th) cells activate and coordinate other cellular components of the immune system. Three types of Th cells have been recognized so far; Th1, Th2 and Th17 cells. In addition, T cells with suppressive function are also found among the CD4$^+$ T cells (Figure 2).

T helper1 (Th1) cells are important for protection against intracellular bacteria and are characterized by secretion of IFN-γ and tumor necrosis factor-α (TNF-α), which stimulate the microbicidal activities of macrophages and dendritic cells. IFN-γ also acts on B cells to stimulate production of opsonizing IgG antibodies. Th1 cells are induced by IL-12, a cytokine produced by natural killer cells, dendritic cells and macrophages (72).

T helper 2 (Th2) cells are characterized by secretion of IL-4, IL-5 and IL-13. These cytokines promote IgE and eosinophil/mast cell-mediated immune reactions, which are protective against helminth infections. Th2 cells are also important for allergic reactions against allergens. Th2 cells are induced by the cytokine IL-4 (72).

T helper 17 cells constitute the latest subgroup of Th cells that has been discovered. Compared with Th1 and Th2 cells, less is yet known about their role. Th17 cells are characterized by secretion of IL-17A (IL-17), which can signal to a variety of cell types (including epithelial cells, endothelial cells, and fibroblasts) to express IL-8, IL-1β, TNF-α, and IL-6 (72). This secretion induces mobilization and activation of neutrophils leading to a neutrophil rich inflammatory response. Th17 cells are thought to be

important in autoimmune diseases but can also protect against extracellular infections, including gram-positive *Propionibacterium acnes*, gram-negative *Klebsiella pneumoniae*, and acid-fast *Mycobacterium tuberculosis* (61, 68, 72).
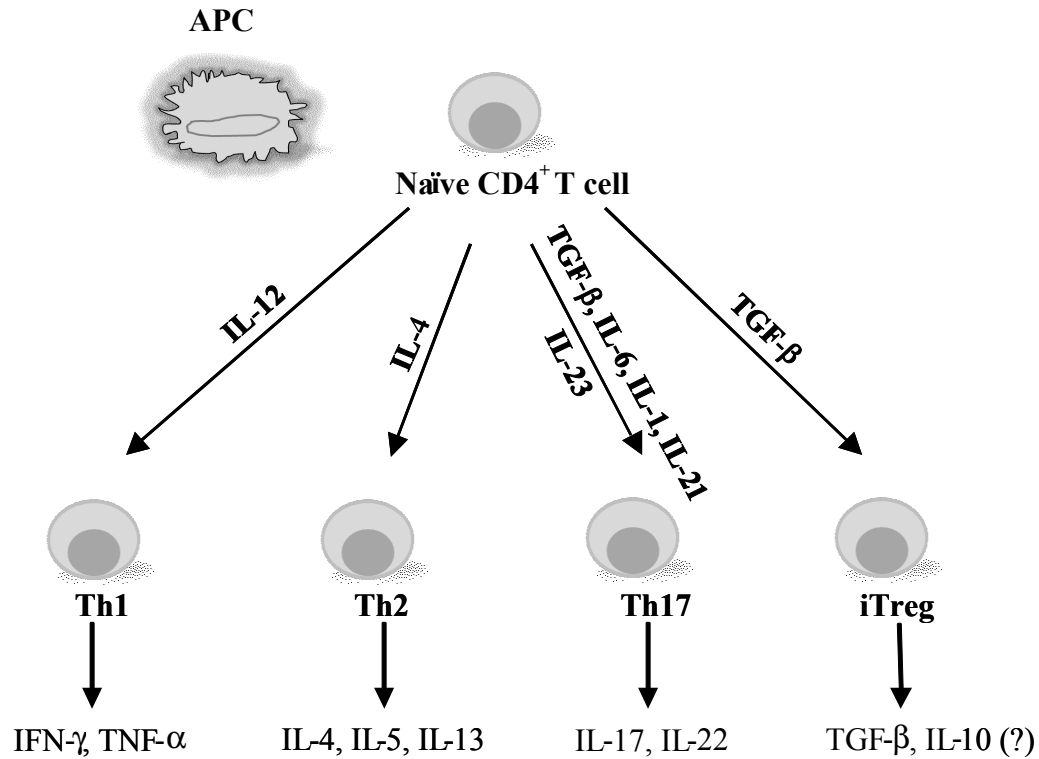

Figure 2. Different CD4^{+} T-cell lineages, their pathways for induction/maintenance and their cytokine secretion profiles in humans.

Naïve cells differentiate to Th17 cells in response to a combination of the immunoregulatory cytokine transforming growth factor-β (TGF-β), proinflammatory cytokines, such as IL-6 and IL-1β, and/or IL-21 (Figure 2) (77, 149). IL-23 appears to be essential for the maintenance of the Th17 phenotype, but does not seem to be directly involved in the induction of these cells (72). IL-23 is a heterodimer composed of a unique p19 subunit together with a p40 subunit shared with IL-12 and this cytokine was first described in early 2000 (107). Data from many earlier studies of the role of IL-12 p40, including studies of immunity to *H. pylori*, need to be reinterpreted based on the new knowledge that the p40 subunit is important for induction of both Th1 and Th17 responses.

Regulatory T cells (Tregs) can suppress T helper cells as well as other cells of the immune system. CD4$^+$ Tregs are now categorized into two main classes; naturally occurring Tregs, which develop their regulatory function in the thymus, and regulatory T cells that gain their regulatory function in the periphery or *in vitro*, i.e. induced Tregs (iTreg) (125). Upon TCR stimulation, a naive CD4$^+$ T cell can be driven to express the transcription factor FOXP3 and become a Treg cell in the presence of TGF-β (71). However, if IL-6 or other proinflammatory cytokines are also present, the naïve T cell differentiate to become a Th17 cell rather than a Treg cell (Figure 2). Treg suppression has been demonstrated to be critically dependent on IL-10 and/or TGF-β *in vivo* (125). However, *in vitro*, suppression has been shown to be contact dependent (14). A link between the contact and cytokine dependent mechanisms of Treg suppression was described when surface bound TGF-β was found to be important for suppression (99). However, these results have later been questioned (110) and the mechanism of Treg mediated suppression remains to be fully clarified. In humans, Tregs have been recognized to be T cells with high expression of the IL-2 receptor α-chain, CD25, and the transcription factor FOXP3 (146). In addition, there are also Tr1 cells, characterized by high IL-10 production (14) and Th3 cells which produce high levels of TGF-β (55), but the relation between the different types of suppressive T cells is still unclear.

T-cell responses to H. pylori infection

Several different T-cell subsets infiltrate *H. pylori* infected mucosa and are likely to contribute to and regulate the immune response to *H. pylori* infection. Murine studies have shown that the gastric inflammation is T-cell dependent, as *H. pylori* does not induce gastritis in T-cell deficient mice (36). Early studies that investigated T- cell responses to *H. pylori* in humans showed that, in healthy controls as well as in *H. pylori*-infected individuals, peripheral blood-derived T cells proliferated in response to stimulation with *H. pylori*-derived antigens, including whole bacteria (67) and crude membranes or cytoplasmic proteins (16). In the human gastric mucosa, *H. pylori* induces recruitment of increased numbers of CD4$^+$ T cells (8, 131).

The mucosal inflammation induced by *H. pylori* has for many years been considered to be primarily of a Th1 type. Freshly isolated lymphocytes and T cell clones

derived from *H. pylori* infected mucosa were shown to secrete IFN-γ (11, 30, 84), and studies in IL-4- and IFN-γ-deficient mice confirm the role of Th1 cells in perpetuating the development of inflammation associated with *H. pylori* infection (127). Recently, several studies have reported that IL-17 is expressed in the stomach of *H. pylori*-infected humans and mice, which suggests that a Th17 response may also be elicited by the infection (22, 95). Both IL-23/p40 and p19 subunits are also upregulated in biopsies of *H. pylori*-infected individuals, indicating that IL-23 may contribute to the Th17 response (22, 72).

However, the T helper cell response to *H. pylori* may be counteracted by the activity of Tregs. Previous studies have shown that stimulation of peripheral blood CD4$^+$ T cells with a membrane preparation of *H. pylori in vitro* results in responses in both infected and uninfected subjects, but that the memory T cells from infected subjects responded less compared to that seen in noninfected controls (83). This nonresponsiveness to *H. pylori* was abolished upon removal of CD4$^+$CD25^{high} Treg cells, indicating the relevance of these Treg cells in suppressing the proliferative response. Furthermore, functionally suppressive FOXP3$^+$ Tregs have been shown to home and accumulate in the *H. pylori*-infected gastric mucosa (37, 83) and increased expression of the suppressive cytokines TGF-β and IL-10 have been observed in infected compared to uninfected mucosa (8, 80). In mice, lack of Tregs has been shown to be associated with increased gastritis and reduced *H. pylori* colonization (115), supporting that under normal conditions, Tregs may contribute to the inability to clear the infection.

B-cell and antibody responses to H. pylori infection

Almost all *H. pylori* infected individuals have antibodies against whole bacteria (101) as well as against a number of purified antigens, including flagellin, urease, lipopolysaccharide (LPS), neutrophil activating protein, the putative adhesin *Helicobacter pylori* adhesin A (HpaA) and membrane protein preparations both in serum and locally in gastric aspirates (92). Furthermore, *H. pylori* infection results in significant increases in plasma cells in the gastric mucosa which primarily produce IgA (91); e.g. in Swedish adults chronically infected with *H. pylori*, more than 40-fold higher numbers of IgA and IgG antibody secreting cells (ASC) were observed in the antral mucosa compared to the stomach of non-infected subjects (91). *H. pylori* specific IgA antibodies

have also been detected in saliva, gastric juice and feces from *H. pylori* infected individuals (49). In addition, the expression of the secretory component on epithelial cells is higher in *H. pylori* infected than in non-infected mucosa (3), suggesting that increased levels of secretory IgA is transported into the lumen during infection. The role of these antibodies is unclear but secretory IgA antibodies may be efficient in neutralizing e.g. urease and VacA as well as in inhibiting adherence of *H. pylori* to the mucosa (27).

Protective immune responses against H. pylori

Despite the T- and B-cell responses induced by *H. pylori*, the infection is chronic in both humans and mice. However, studies in mouse models of *H. pylori* infection show that the infection can be prevented, and established infections eradicated by many different types of immunizations. There is clear evidence that CD4$^+$ T cells are required for immunization induced protective immunity against the infection. Thus, mice lacking MHCI, but not MHCII, are protected against *H. pylori* infection after immunization, suggesting that CD4$^+$ but not CD8$^+$ T cells are required for effective immunity (38, 108). Although there is strong evidence that IFN-γ is important for development of gastritis and for controlling the bacterial colonization in unimmunized mice (43, 123, 127), the role for IFN-γ in immunization induced protection is less clear. Although IFN-γ deficient mice fail to develop protection after immunization in some studies (6), immunization induced protection has been demonstrated in such mice in other reports (43, 123). Furthermore, studies showing that IL-12p40 knockout mice, which are deficient in IL-23 as well as IL-12 production, are not protected against *H. pylori* infection after immunization (6, 43), suggest that the Th17 cell subset may also play a role in protection against *H. pylori* (6, 43). More recent studies confirm this notion, since IL-17 neutralization or neutrophil depletion inhibits vaccine-induced reduction of *H. pylori* colonization (32, 144). Some studies also suggest that Th2 responses are important for protection (96), although this remains controversial (2, 6).

The role of antibodies for immunity against *H. pylori* is less clear than the role of CD4$^+$ T cells. Czinn *et al.* showed that preincubation of bacteria with urease specific monoclonal antibodies decreased the bacterial infectivity (29) and Nystrom *et al.* observed a direct relation between protection and IgA levels in the stomach (103).

Furthermore, there are some indications that antibodies may reduce the gastritis because mice lacking antibodies have more gastritis and also clear the infection quicker than wild-type mice (7). On the other hand, knockout studies have shown that mice lacking antibodies are equally well protected against challenge as wild-type mice (38, 132). In addition, it has been demonstrated that the prevalence of *H. pylori* infection does not differ between IgA deficient and normal Swedish individuals (19). However, patients with IgA deficiency are at increased risk of developing gastrointestinal carcinomas (41). A recent study has also shown that gastric adenocarcinoma patients have decreased production of gastric IgA antibodies which may have an impact on the development of disease (114). However, it is not known whether this low IgA production is a cause or effect of development of gastric malignancy.

H. pylori infection in children

The majority of *H. pylori* infections are acquired during the first decade of life (40, 94). A major risk factor for acquisition of *H. pylori* infection is low socioeconomic status of the family during childhood, e.g. high numbers of persons in a household (person to person transmission), sharing of bed, poor sanitation and personal hygiene (fecal-oral transmission) (70).

H. pylori-induced diseases in children

Although *H. pylori* is etiologically associated with chronic active gastritis, peptic ulcers and gastric adenocarcinoma, duodenal ulcers are usually not seen before young adulthood or at mid life and gastric cancer is primarily seen in people over the age of 50 (13, 52). Thus, although children develop chronic gastritis, this is generally an asymptomatic condition and symptomatic diseases are infrequent. At present, there is no evidence to suggest a link between *H. pylori* gastritis and pain in the abdomen in children (13).

T-cell responses to H. pylori in children

There are a few reports describing the lymphocyte subsets present in the gastric mucosa of children infected with *H. pylori*. These studies show that the stomach mucosa is infiltrated by increased numbers of mononuclear cells and CD3^{+} T cells in *H. pylori*

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infected compared to uninfected children (20, 58). It has been also reported that elevated levels of IFN-γ, IL-17, IL-12 and IL-8 are found in infected mucosa of children (20, 86). In contrast, the levels of the Th2 cytokines IL-4 and the regulatory cytokine IL-10 do not differ between *H. pylori* infected and uninfected children (86). In a recent report, infiltration of cytotoxic T lymphocytes was found to be more prominent in children with a high risk for gastric cancer than in children from a low-risk population (13). The predominance of cytotoxic T lymphocytes in risk group children suggests that they may predispose to more severe damage in such high-risk populations. However, some investigators (58, 148) have reported that *H. pylori* associated gastritis in children is reduced compared with that of adults, possibly as a result of the presence of high numbers of Tregs in the gastric mucosa of children (58). It has been suggested that local Treg cell activity in children down-regulates the Th1-mediated inflammation typical of *H. pylori*-associated gastritis in adult hosts. This suppression is consistent with the 10-fold lower level of IFN-γ found in the gastric mucosa of children compared with that of adults (20).

Antibody responses in children

There are very few studies on antibody responses to *H. pylori* in young children, both in high endemic and low endemic countries. In a study in a rural area in Egypt, 13% of the children at 7-9 months of age were positive for IgG antibodies against *H. pylori* urease using a commercial ELISA test (12). By 18 months of age seropositivity had increased to 25%, whereas 88% of the mothers were positive using the same assay. When IgG antibody responses to *H. pylori* were compared in children and adults in Mexico (142), it was found that 37% of the children below 10 years of age were seropositive (IgG titers against whole cell antigen) as compared to 89% of the adults. Furthermore, a considerably lower frequency of the children in this study had significant IgG antibody levels against CagA (47%) and urease (16%) than the adults (79% and 59% respectively). In recent studies in East Europe, where the prevalence of *H. pylori* also is high (65), 79% of 8-16 year old Lithuanian children with gastritis or duodenal ulcers were histologically positive for *H. pylori*. Of these positive children, 57% had increased IgG antibody levels against *H. pylori* as detected using a low molecular weight antigen preparation and adult

cut-off levels in the ELISA test (65). However, adult cut-off levels may sometimes result in lower sensitivities in children than in adults (23, 28, 31), which could explain the comparatively low seropositivity in this study. Studies of IgG antibody responses to *H. pylori* in asymptomatic school children in Germany with a mean age of 11 years reached a seropositivity of 20% using ELISA (18). A significantly higher level of seropositivity was observed among children of Turkish (38%) compared to German nationality (14%) (18). A very low level of responders to *H. pylori* have also been observed in Swedish children, with only 3% being seropositive at 11 years of age (54).

Role of breast feeding for protection against H. pylori infection

A role of breast milk antibodies for protection against *H. pylori* infection has been suggested. In a study in Gambian infants, intake of high levels of anti-*H. pylori* IgA antibodies in breast milk was associated with delayed age of onset of *H. pylori* infection (138). It has also been suggested that urease antibodies may protect against colonization with *H. pylori* during infancy (138). Furthermore, human milk has been shown to inhibit the adherence of *H. pylori* to a gastric adenocarcinoma cell line by 50-70% (26) and lactoferrin from human breast milk binds to *H. pylori* LPS leading to its inactivation (9). In a recent meta-analysis on the role of breast milk for protection against *H. pylori*, it was concluded that breastfeeding is protective, especially in LMIC, probably due to high levels of specific IgA antibodies in mother's milk (24).

In this thesis we have studied the development of B- and T-cell immune responses to *H. pylori* in young infants in a highly endemic area for *H. pylori* and the role of maternal antibodies and the childrens' own immune responses for protection against infection. We have also compared immune responses to *H. pylori* in adults in a high and low endemic area.

AIMS OF THE STUDY

The overall aim of this thesis was to analyze humoral and cellular immune responses to *H. pylori* in children and adults living in a developing country with a high prevalence of *H. pylori* infection. The specific aims were:

- to analyze and compare immune responses to *H. pylori* in infected Bangladeshi and Swedish adults.

- to establish simple approaches for detection of *H. pylori* infection in infants and young children in a developing country setting.

- to determine the incidence of *H. pylori* infection in Bangladeshi children during their first two years of life in relation to season, blood group, nutritional status and co-infection with enteric pathogens.

- to study the development of systemic and mucosal antibody responses to *H. pylori* infections acquired during infancy and early childhood.

- to evaluate the relation between maternal antibodies and acute *H. pylori* infection during infancy.

- to determine if certain T-cell responses (Th1 and Th17) may contribute to the immune responses against *H. pylori* in young children and adults.

MATERIALS AND METHODS

Study sites

The studies described in this thesis were performed in two different areas in Bangladesh; in Dhaka city and in Mirpur, 10 miles west of Dhaka city center, as well as in Gothenburg, Sweden. Bangladesh, on the Northern coast of the Bay of Bengal, has a subtropical monsoon climate characterized by high humidity and wide seasonal variations in rainfall and temperatures. Three seasons are generally recognized: a hot, humid summer from March to June; a cooler, rainy monsoon season from June to October; and a cool, dry winter from October to March.

For comparison of immune response against *H. pylori* in high and low endemic areas (Paper I), we recruited infected patients with duodenal ulcers (DU) and asymptomatic (AS) carriers without symptoms of the infection in Dhaka city and AS participants and *H. pylori*-negative subjects in Gothenburg (Table 2). The Bangladeshi AS subjects were recruited at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) and DU subjects were recruited from in and around Dhaka city via the Dhaka Medical College Hospital. Swedish AS and *H. pylori* negative participants were recruited among blood donors at the Sahlgrenska University Hospital (SUH) in Gothenburg.

For analysis of the epidemiological features of *H. pylori* infection during the first two years of life and for studies of the development of immune responses against the infection (Paper II, III), samples were collected from children participating in a prospective community based birth cohort study conducted in the Mirpur field area in Bangladesh from April 2002 to October 2004 (112) (Table 2). The area of Mirpur is around 90 sq km and is densely populated with 2.5 millon inhabitants, corresponding to about 20% of the population in Dhaka city. We chose the Mirpur site for our studies since it is representative of low to middle income community, where our laboratories have experience in carrying out a large number of field and laboratory based studies over the last 15 years. For the studies of T-cell cytokine responses to *H. pylori* (Paper IV), children and adults were recruited from the same field area (Table 2).

Participants and collection of specimens

Studies of B- and T-cell responses in adults (Paper I)

To compare B- and T-cell immune responses to *H. pylori* in high and low endemic areas, adult *H. pylori*-infected DU and AS participants were recruited to the study in both Bangladesh and Sweden (Table 2). Due to the high prevalence of *H. pylori* infection in Bangladesh, it was difficult to recruit uninfected Bangladeshi individuals. Instead, uninfected Swedish participants served as controls. AS subjects or uninfected controls had no signs of ulceration at the time of endoscopy and did not have any gastrointestinal symptoms or illness during the preceding three weeks. The DU patients had at least one visual ulcer in the duodenum at the time of endoscopy. None of the participants were on any medication during the preceding three weeks before enrollment.

Table 2. Characteristics of participants in the different studies.

Papers	No of participants	Ages	Study sites	Samples collected
Paper I	15 [a]	20-60 years	Dhaka (AS)	Blood, stool, antral and duodenal biopsies
	24	18-42 years	Dhaka (DU)	
	14	31-61 years	Gothenburg (AS)	
	16	22-63 years	Gothenburg (Hp⁻)	
Paper II, III	238	0-24 months	Mirpur, Dhaka	Blood, stool
	44	18-35 years	Mirpur, Dhaka	Blood, breast milk
Paper IV	16	6-12 months	Mirpur, Dhaka	Blood, stool
	10	3-5 years	Mirpur, Dhaka	Blood, stool
	15	19-32 years	Mirpur, Dhaka	Blood, stool
	1	25 years	Dhaka (Hp-)	Antral biopsies
	7	46-89 years	Gothenburg (Hp⁺)	Antral biopsies
	6	57-77 years	Gothenburg (Hp⁻)	Antral biopsies

[a] Six of these individuals were also included in Paper IV for analysis of mRNA levels.

Blood samples from Swedish and Bangladeshi subjects and stool samples from the Bangladeshis were collected prior to enrollment in the study for preliminary *H. pylori* diagnosis (see below). Biopsies were collected from both the antral and the duodenal mucosa of all participants by gastroduodenal endoscopy. Biopsies from the antrum and duodenum respectively were embedded in optimal cutting temperature compound (OCT), immediately snap-frozen, and used for immunohistochemistry (IHC). Biopsies from each site were fixed in formalin and used for histological examination. From a subset of the participants, 1-2 biopsies were stored at -70°C for protein analysis using a saponin extraction method (15). The remaining biopsies from each site were pooled and used for isolation of lymphocytes and subsequent flow cytometric (FCM) analyses. Blood specimens were collected from all participants on the day of endoscopy.

Samples collected in Bangladesh for this study (Paper I), (except for the samples used for flow cytometry) were shipped to Sweden in dry ice for analysis and kept frozen at all times; control experiments were performed in Bangladesh and Sweden to verify that similar results were obtained before and after transportation.

Birth cohort studies (Paper II, III)

For the epidemiological and immunological *H. pylori* studies in young children from birth up to 2 years of age, 238 children were enrolled (Table 2, Paper II) (112). We also analyzed immune responses against *H. pylori* infection, reinfection and spontaneous eradication in these children (Paper III). The general health status of the children at the time of inclusion in the study was assessed by a study physician.

Fecal specimens were collected from each child every month and blood at three month intervals (Figure 3). In addition, cord blood was collected just after birth. Samples collected every sixth month, or in a subset of children every third month, were tested for *H. pylori* using a stool antigen test. Blood samples from the mothers were collected every 6 month and breast milk every third month. All samples were transferred cold from Mirpur to the laboratory at ICDDR,B and stored at -70°C until laboratory tests were performed in the same laboratory.

	Time →										n
Month	0 1	3	6	9	12	15	18	21	24	
Ch stool	**+** +	**+**	+	**+**	+	**+**	+	**+**		50/238
Ch blood	+	+	+	+	+	+	+	+	+	50
Mo blood	+									44
Mo breast milk	+	+	+	+	+					20

Figure 3. Schematic diagram of time points for analysis of specimens from children (Ch) and mothers (Mo) in the birth cohort studies (Paper II & III). Bold symbols (**+**) indicate time points when specimens from all children were analyzed (n=238). "+" indicates time points when samples from selected children (n=50) and mothers (n=20-44) were analyzed.

Cytokine studies in children and adults (Paper IV)

For analysis of cytokine production by T cells in response to stimulation with *H. pylori* antigens, *H. pylori*-positive and *H. pylori*-negative adults (19-32 years), children (3-5 years) and infants (6-12 months) were recruited in the Mirpur field area in Bangladesh (Table 2). Only AS participants who did not have any symptoms of the infection and did not have any illness during the preceding three weeks before participation were enrolled. Blood cells were stimulated with different antigens and collected culture supernatants were analyzed either in Gothenburg or in Dhaka. Some randomly selected supernatants were analyzed in both Sweden and Bangladesh as a control experiment and we observed similar results in the two different laboratories. Stool and blood samples were collected once from each volunteer.

Detection of *H. pylori* infections (Paper I-IV)

For preliminary diagnosis of *H. pylori* infection in Swedish and Bangladeshi participants, plasma samples were screened at the respective site for presence of *H. pylori*-specific IgG antibodies using an in-house enzyme-linked immunosorbent assay (ELISA) (15, 92). In Bangladeshi children and adults, the *H. pylori* status was also analyzed using a monoclonal antibody based test for detection of *H. pylori* specific antigen in the stool (Amplified IDEIA Hp StAR, Dako, Denmark). For some of the Bangladeshi and Swedish adults (Paper I), infection was later confirmed by culture of biopsies on horse blood-

Columbia Iso agar plates at 37°C under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2); after 3 days of culture, *H. pylori* bacteria could be detected from the vast majority (>90%) of *H. pylori* seropositive participants but not from any of the seronegative individuals. When *H. pylori* bacteria could not be cultured from seropositive individuals, infection was confirmed by stool antigen test (in Bangladesh) or by urea breath test (in Sweden).

Analysis of *H. pylori* specific antibodies (Paper I-IV)

Titers of *H. pylori*-specific IgA and IgG antibodies in plasma, breast milk, stool extracts and biopsy extracts were determined by the in-house ELISA tests as previously described (15, 92). A membrane protein preparation (MP) from the *H. pylori* strain Hel 305 isolated from a Swedish duodenal ulcer patient was used as coating antigen. For some serological analyses (Paper I), MPs from Bangladeshi *H. pylori* strains were also used. The MPs were prepared by sonication of the bacteria followed by differential centrifugation, as previously described (1). The total amounts of IgA in mucosal biopsy extracts and plasma were determined using ELISA (15).

Cell isolation (Paper I & IV)

For analysis of lamina propria lymphocytes (LPLs), cells were isolated from the biopsies by incubation in EDTA/DTT and subsequent collagenase/DNase treatment, as previously described (83). Initial experiments showed that this cell isolation protocol gave a maximal yield of cells, with little of the epithelium remaining in the lamina propria fraction, and that the isolation procedure had only marginal effects on the expression of different cell surface markers.

For analysis of T-cell cytokine responses, peripheral blood mononuclear cells (PBMCs) were isolated from blood collected in heparinized vials by gradient centrifugation on Ficoll-Isopaque. Plasma collected from the top of the Ficoll gradient was stored in aliquots at $-20^{o}C$ for antibody assays.

In additional experiments, CD4^{+} T cells were depleted from the PBMCs to determine the T-cell subset responsible for cytokine production using Dynal (CD4^{+}) beads according to the instructions provided by the manufacturers. CD4^{+} T cells were

also isolated by detaching the T cells from the beads according to the instructions provided.

Flow cytometry (Paper I & IV)

LPLs and PBMCs were stained with fluorescently labeled antibodies. Cells were fixed in formaldehyde before FCM analysis, which was performed on FACSCalibur instruments in both Bangladesh and Sweden. Calibrite beads (BD Pharmingen) were used to achieve comparable settings on the two machines used in Bangladesh and Sweden. Control cell samples were run on the two machines by the same operator to ensure consistent results.

The FCM data were analyzed with FlowJo software (Tree Star Inc.). The number of cells isolated from pooled biopsies from each individual was estimated by multiplying the frequency of positive cells detected by FCM with the total number of lymphocytes isolated from each biopsy. FCM data from analysis of PBMCs were reported as frequencies of cells.

Histopathology (Paper I)

For histological analysis of mucosal samples, biopsies collected from the antral and duodenal mucosa from both Swedish and Bangladeshi adults were fixed in formalin, embedded in paraffin and routinely processed for histology at the pathology unit at SUH in Sweden. The inflammation was graded on a scale from 0-3, corresponding to none, mild, moderate and severe inflammation, according to the Sydney system (111).

Immunofluorescence (Paper I)

To analyse the numbers of Treg in mucosal biopsies, CD4^{+} T cells expressing the Treg marker FOXP3 were stained using fluorescent labeled anti-FOXP3 and anti-CD4 antibodies (DAKO, Glostrup, Denmark), as described (37). The numbers of positive CD4^{+}FOXP3^{+} cells in the lamina propria in each section were counted using a Zeiss Axiovert 100TV fluorescent microscope. The total areas of the sections were measured using Zeiss Axiovision software and the number of positive cells was expressed per square millimeter of total tissue area.

Anthropometric analyses (Paper II)

To determine the relationship between malnutrition and *H. pylori* infection in children, the nutritional status of the children in the birth cohort study was assessed at birth and at 1, 6, 12, 18 and 24 months of age. Children who were undernourished (<-2SD of weight for age) or stunted (<-2SD of height for age) were identified by comparison with the weight and height of the National Center for Health Statistics reference population of the same age and sex.

Identification of other enteric infections (Paper I-III)

The monthly stool samples collected from the Bangladeshi participants in the birth cohort and adult studies were analyzed for enterotoxigenic *Escherichia coli* (ETEC) using GM1-ELISA for LT and ST expression and dot blot analysis of colonizing factor antigens (126). The stool samples were also cultured for other enteric pathogens, e.g. *Vibrio cholerae* O1/O139, *Salmonella*, *Shigella* and *Campylobacter* spp., as well as analyzed for rotavirus by ELISA and tested by direct microscopy to detect cyst and vegetative forms of parasites and ova of helminths.

Blood grouping (Paper II)

Blood specimens collected from the children in the birth cohort study were typed for ABO blood group and Rh factors using a slide agglutination procedure using antisera from Biotech Laboratories, Suffolk, UK.

Recording of breast feeding practices (Paper II-III)

Mothers of children participating in the birth cohort studies were asked every month by the field attendants about their breast feeding practices and the data were recorded. All children were breastfed for at least 6 months. Children who during the initial 6 months of life received breast milk only were termed as 'exclusively breast fed' and children who in addition were also fed with water, honey or sugar syrup were designated "partially breast fed". After 6 months of age, children were breast fed in varying degrees up to 2 years.

T-cell stimulation assays (Paper IV)

For analysis of cytokine responses, isolated PBMCs and PBMCs depleted of $CD4^+$ T cells were incubated in 96-well U-bottomed tissue culture plates in DMEM medium (Invitrogen AB, Sweden) with 5% human serum and 1% gentamycin at 37^oC in 5% CO_2 for 5 days. PBMCs were stimulated with *H. pylori* MP (Hel 305 MP, 1 µg/ml), and phytohemagglutinin (PHA, 1 µg/ml) for 5 days. In addition, $CD4^+$ cells were stimulated with anti-CD3/CD28 coated expansion beads at a bead to cell ratio of 1:1. After 5 days, 100 µl supernatants were collected from all cultures and the samples were frozen immediately in aliquots at -70^oC until assayed for cytokines.

Analysis of chemokines and cytokines (Paper I & IV)

For analysis of different chemokines in mucosal tissues, proteins were extracted from biopsies by incubation of the tissue in 2% saponin solution overnight at 4°C (15). The concentrations of the chemokines IP-10 (CXCL9), MIG (CXCL10) and IL-8 (CXCL8) in the extracts were determined by the cytometric bead array (CBA, BD Pharmingen). The concentration of MDC (CCL22) was measured using ELISA (R&D Systems). The total protein concentrations in the extracts were measured by the Bio-Rad protein assay (Hercules, CA).

To determine the levels of different cytokines in culture supernatants (Paper IV), we performed ELISAs for IL-17A (ebioSciences, USA) and IL-13 (R&D, Sweden) according to the instructions provided by individual manufacturers and we used CBA for analysis of IFN-γ, TNF-α, IL-10, IL-5, IL-4 and IL-2 (BD Pharmingen).

Cytokine gene expression analysis (Paper IV)

RNA was isolated from RNALater-stabilized human tissue specimens Qiagen's RNeasy Mini kit and used for cDNA synthesis using the Omniscript Reverse Transcription kit (Qiagen). The relative expression of IL-17 and IFN-γ mRNAs was subsequently determined with quantitative real-time PCR gene expression assays from Applied Biosystems using HPRT as an internal reference gene. Gene expression changes data were analyzed using the ΔΔCt method, calculating fold change of each gene, normalized to the reference gene and relative to an external calibrator sample.

Data analyses (Paper I-IV)

Data were analyzed using the statistical programs GraphPad PRISM 4.0, EpiInfo version 2000, SPSS for Windows (Version 10.00) and SigmaStat 3.1 (SPSS Systat software, Inc). Paired samples were assessed by the Wilcoxon signed rank test, non-paired samples by the Mann-Whitney U-test and proportion of responses using the Chi-square or the Fisher exact test. In addition, the Chi-Square or Fisher exact tests were also used for comparison of epidemiological differences in *H. pylori* positive and negative subjects (Paper II, III). For multiple comparisons, Kruskal-Wallis test with Dunn's post hoc test was used (Paper I). *P* values <0.05 were considered to be statistically significant.

RESULTS AND COMMENTS

Comparison of humoral and cellular immune responses to *H. pylori* in infected Bangladeshi and Swedish adults (Paper I)

Most studies of immune responses to *H. pylori* have been performed in developed countries. However, nowadays *H. pylori* infection is becoming rare in most of the developed world. Therefore, we initiated a study in Bangladesh, where the prevalence of *H. pylori* infection is very high, to determine if immune responses to *H. pylori* may be different in a highly endemic country to in a low endemic country. To this end, we have analyzed immune responses in adults living in Bangladesh and Sweden. We also compared immune responses to *H. pylori* in AS carriers and in DU patients in Bangladesh to investigate if there are immune responses that may be related to development of DU disease.

Comparison of immune responses to MP antigens prepared from Bangladeshi and Swedish H. pylori strains for use in immunoassays (Paper I)

To establish an ELISA assay that could be used for determination of antibody responses in Swedish and Bangladeshi subjects, a membrane protein (MP) preparation from the *H. pylori* strain Hel 305 isolated from a Swedish DU patient was compared with MPs from several different *H. pylori* strains isolated from Bangladeshi participants. The Hel 305 MP preparation has been extensively used in studies in Sweden (91, 92, 114, 141). Sera from both Bangladeshi and Swedish *H. pylori*-infected participants reacted with both the Bangladeshi and Swedish strain MPs, but the antigen prepared from the Swedish strain gave consistently higher antibody titers than the MP prepared from Bangladeshi strains (exemplified in Figure 4 for strain D94 isolated in Bangladesh). Since we observed 3-6 fold higher *H. pylori*-specific IgA antibody titers against Swedish MP in sera from both Bangladeshi and Swedish asymptomatic participants than against the Bangladeshi MP tested, we chose Hel 305 MP as coating antigen in our studies. The same MP was also used to study T-cell responses (Paper IV).

By performing the ELISA tests on sera from *H. pylori* infected subjects, we also observed that the titers of *H. pylori*-specific IgA (Figure 4) and IgG (not shown) antibodies were about twofold lower in the Bangladeshi compared to in the Swedish samples. As most of the Bangladeshi participants included in the study were probably

less well nourished, the nutritional status may explain at least some of the serological differences observed between Bangladeshi and Swedish participants.
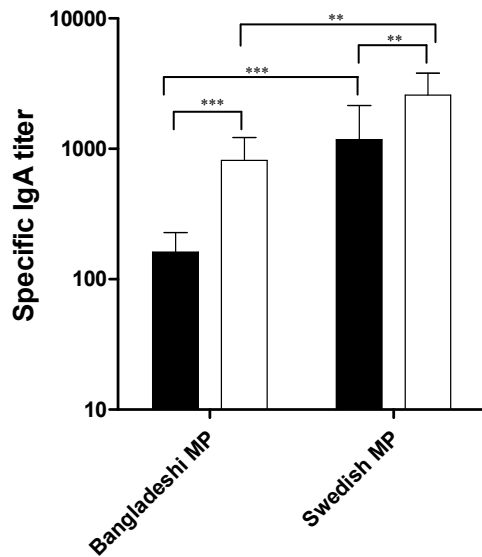

Figure 4. Comparison of ELISA titers against MP prepared from Bangladeshi (strain D94) and Swedish (strain Hel 305) *H. pylori* bacteria, respectively (geometric mean values + SEM). IgA antibody titers in sera from adult Bangladeshi (black bars; n=10) and Swedish (white bars; n=20) participants are shown. (**P<0.01, ***P<0.001).

Mucosal inflammation in Bangladeshi and Swedish adults (Paper I)

Our observation of different levels of serum antibody responses to *H. pylori* in the different populations encouraged us to also investigate the mucosal inflammatory responses against *H. pylori* in Swedish and Bangladeshi subjects. This was done by both histological analysis of biopsy material and analysis of antibodies extracted from the biopsies. As >90% of Bangladeshi adults are infected by *H. pylori*, it was very difficult to recruit uninfected Bangladeshis and we were able to recruit only one uninfected control in Bangladesh (not included in Paper I but shown in this thesis). Instead, we primarily compared immune responses in *H. pylori*-infected Bangladeshi subjects with responses in Swedish infected and uninfected participants.

We isolated similar numbers of LPLs from antral biopsies from Bangladeshi and Swedish *H. pylori*-infected AS individuals (Figure 5a), indicating a comparable infiltration of cells into the antral mucosa of subjects from both countries. However, significantly lower numbers of LPLs were isolated from uninfected gastric mucosa of *H. pylori*-negative Swedes; similarly low cell numbers were isolated from the uninfected Bangladeshi control individual, supporting the observation that the higher lymphocyte numbers observed in the antrum of infected individuals are a consequence of *H. pylori*

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infection in both populations. In contrast, higher numbers of LPLs were isolated from duodenal biopsies from Bangladeshi than from Swedish participants (Figure 5b), suggesting the presence of more duodenal inflammation in the Bangladeshis. On the other hand, the number of LPLs isolated from the duodenum were very similar in Swedish AS carriers and *H. pylori*-uninfected participants (Figure 5b). Histological examination of antral and duodenal biopsy material as well as analysis of mucosal chemokine levels also to a large extent supported the presence of similar levels of inflammation in the antrum, but higher levels in the duodenum of Bangladeshi compared to Swedish participants. The presence of higher inflammation in the duodenum of Bangladeshi individuals may reflect recruitment of both *H. pylori*-specific and non-specific lymphocytes to the duodenum.
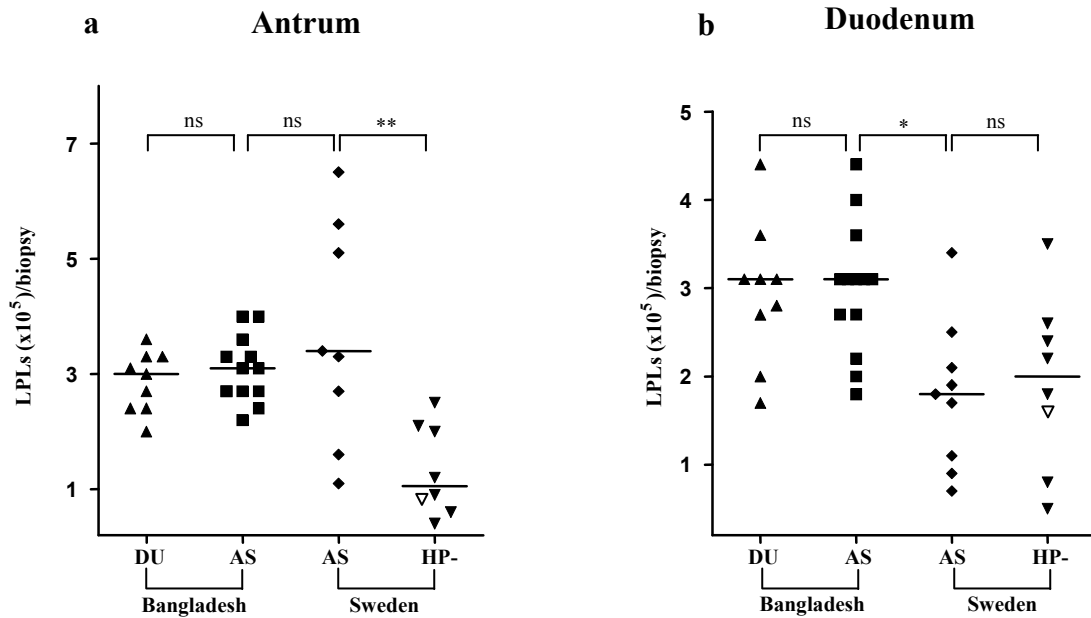

Figure 5. Comparison of numbers of lamina propria lymphocytes isolated from antral and duodenal biopsies from Bangladeshi *H. pylori* infected DU (▲) and AS (■) and Swedish *H. pylori* positive AS (♦) and *H. pylori* negative (▼) subjects. The median values are indicated by horizontal lines. Open triangle (∇) shows the level of LPLs in the Bangladeshi *H. pylori* uninfected control. (*P<0.05, **P<0.01; ns: not significant).

Mucosal B- and T-cell responses in Bangladeshi and Swedish adults

We also studied the relative contributions of different lymphocyte subsets to the inflammation. Mucosal cells were analyzed by FCM and in addition, we analyzed the levels of *H. pylori*-specific and total IgA in mucosal tissue extracts by ELISA.

Comparable numbers of CD19$^+$ B cells and similar levels of *H. pylori*-specific IgA antibodies were observed in the antrum of *H. pylori* infected Bangladeshi and Swedish participants. The numbers of CD4$^+$ and CD8$^+$ T cells and FOXP3$^+$ Tregs were also similar in the gastric mucosa of these study groups. Higher numbers of CD19$^+$ B cells, *H. pylori* specific IgA antibodies and CD4$^+$ T cells and similar levels of CD8$^+$ T cells were isolated from the stomach of infected compared to uninfected Swedes. In contrast, about 3-fold higher numbers of CD19$^+$ B cells and 12-fold increased levels of *H. pylori*-specific IgA antibodies were found in the duodenum of the Bangladeshi subjects compared to in the Swedish volunteers. However, the numbers of T cells were similar in duodenal biopsies from the different study groups. The numbers of CD19$^+$ B cells, CD4$^+$ T cells, CD8$^+$ T cells and levels of specific IgA were low and comparable in the duodenum of both *H. pylori*-infected and uninfected Swedish participants.

Taken together, our results show a lower systemic antibody response to *H. pylori*, but no major differences in immune responses in the stomach mucosa, in the Bangladeshis compared to the Swedish adults. On the other hand, increased inflammation with higher numbers of B cells and higher levels of both *H. pylori* specific and total IgA were observed in the duodenum of the Bangladeshis, which may be due to considerably higher exposure to other gastroenteric infections in Bangladesh.

Studies of *H. pylori* infection in infants and young children in Bangladesh from birth to two years (Paper II & III)

Since we found comparable levels of immune responses to *H. pylori* in Bangladeshi and Swedish adults who were chronically infected, we were interested in also studying immune responses against acute infection in young children in Bangladesh. Before initiating studies of immune responses in this age group, we determined the prevalence of *H. pylori* infection in children in the selected Mirpur study area. This was done by following a cohort of children from birth up to 2 years of age for onset and duration of *H. pylori* infection. To allow demonstration of *H. pylori* infection in young children in this poor resource area, simple detection methods for *H. pylori* were used. Traditional diagnostic methods, e.g. endoscopy and UBT, are not practically feasible in this area since endoscopy of children for collection of biopsies is not possible and samples for UBT need to be sent to well-equipped laboratories abroad. Therefore, we used a

combination of tests, e.g. analysis of IgA and IgG antibodies in serum and *H. pylori* antigen in stool.

Determination of H. pylori infection during early childhood using stool antigen test and serology (Paper II & III)

Fifty to 60% of the children in the birth cohort were found to be positive for *H. pylori* by two years of age using stool antigen test and serology. Thus, we had established that *H. pylori*-specific ELISA titer of ≥100 for the IgA isotype and ≥1000 for the IgG isotype signified positive serology after titration of sera from stool antigen positive and negative children. However, we found that usage of IgG serology for determination of *H. pylori* infection in infants is less suitable. Thus, when analyzing the kinetics of the antibody responses in the children, we found that the serum IgG titers decreased rapidly from the levels in cord blood during the initial 6 months of life, whereafter they increased in all groups during the two year study period. IgG titers also lasted during long periods of time after eradication of the infection (Paper III).

To evaluate to which extent maternal IgG antibodies had been transferred to the children through the placenta, we compared the IgG titers in mother's blood and in cord blood. A very high correlation was observed (P<0.0001; Figure 6); supporting transplacental transfer of *H. pylori* specific IgG antibodies. No IgA titers were observed in cord blood. The drastic drop in serum IgG observed during the first 6 months of life also supports a maternal origin of the serum antibodies during early infancy.
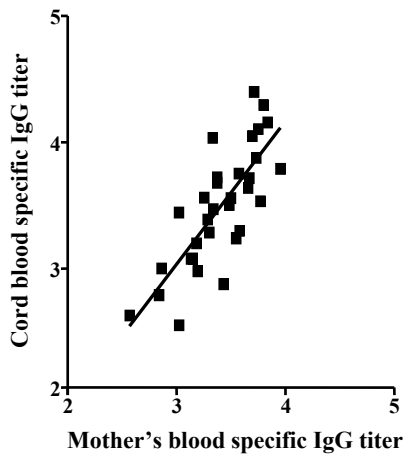

Figure 6. Correlation between IgG titers against *H. pylori* MP in mother's blood and in cord blood (n=32). (P<0.0001, r= 0.78, Spearman correlation test).

In contrast to the serum IgG titers, the serum IgA titers in the children were low during the initial 6 months and then gradually increased. Furthermore, IgA titer rises in the stool antigen negative children were very modest in serum during the study period. In addition, IgA titers rose rapidly after infection and decreased if the infection was cleared (Paper III). We also observed a highly significant correlation between serum IgG and IgA levels in relation to *H. pylori* infection (Spearman r = 0.73; P<0.0001) at 24 months of age. Since IgA is not transferred from the mother during fetal life, maternal antibodies do not interfere with the childrens' serum IgA levels during the initial 6 months of life. Therefore, measurement of serum IgA may be a suitable method for detection of *H. pylori* infection in young children.

We also compared *H. pylori*-specific IgA and IgG serum titers in Bangladeshi infants (0-12 months), children (>12-24 months) and adults (18-60 years) (Paper I-III; Figure 7). Our results show that the levels of antibodies increase with age with the highest increases seen for IgG early during childhood and for IgA between childhood and adulthood. Adults have about 15-fold higher IgA titers and 3-fold higher IgG titers than infants (Figure 7). In the adult subjects, there was no correlation between age and antibody levels.
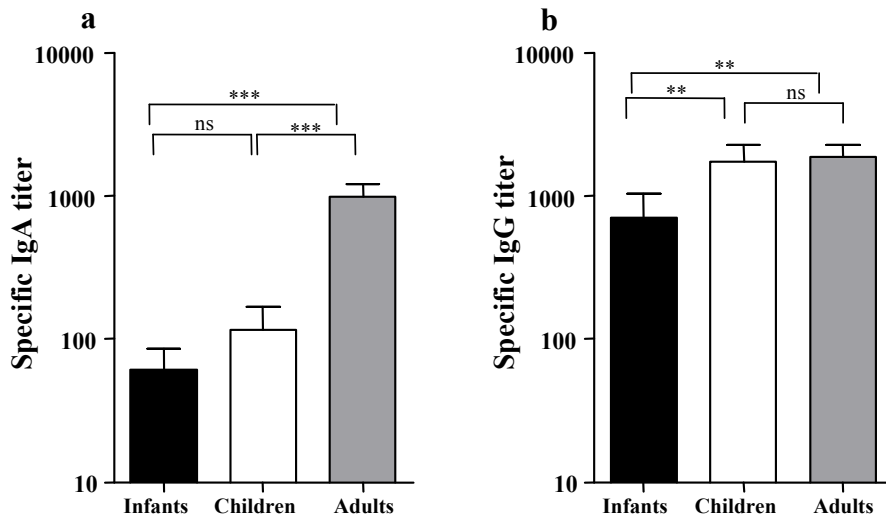

Figure 7. Comparison of levels of IgA (a) and IgG (b) antibodies against *H. pylori* MP (Hel 305 MP) in sera of Bangladeshi *H. pylori* infected infants (0-12 months for IgA, 6-12 months for IgG; black bars, n=15), children (>12-24 months; white bars, n=30) and adults (18-49 years; grey bars, n=32). (**P<0.01, ***P<0.001; ns: not significant).

We also compared the sensitivity, specificity, and positive predictive value of the stool antigen test in relation to serology (IgA and IgG) and vice versa. Initially, we only compared results from the stool antigen test and IgG serology for detection of *H. pylori* (Paper II). However, due to problems with transplacental IgG, we later also included comparison of results from the stool antigen test and IgA serology (Paper III). When comparing IgG responses in serum with the responses seen with the stool antigen test, using specimens collected between 6 and 24 months of age, the sensitivity was 93% at 18 months and 100% at any time point during the study period. When IgA responses in serum were used as the standard and compared to results obtained with stool antigen test, the sensitivities of the serology was 75% at 18 months and 87% at any time point between 0 and 24 months (Table 3). The specificities of serum IgA were higher than serum IgG in relation to stool antigen test (73-98% for IgA and 47-77% for IgG at different time points). The limitation of the stool antigen test is that it may give falsely negative results and hence be less sensitive than serology. However, when analyzing several stool specimens from the same child the detection rate of *H. pylori* was considerably higher. Hence, we recommend collection of at least 2 stool specimens when using the stool antigen test for detection of *H. pylori* in young children. In conclusion, IgA serology in combination with a stool antigen test may be the most accurate and feasible approach for detection of *H. pylori* in settings with less resourceful laboratories.

Table 3. Comparison of stool antigen test and IgA serology for detection of *H. pylori* at various time points in children in the Bangladeshi birth cohort (Paper II & III).

	Age (n=50)[a]	Sensitivity	Specificity	Positive predictive value
6 mo	Stool vs serology (%)	50	94	25
	Serology vs stool (%)	25	98	50
12 mo	Stool vs serology (%)	57	89	67
	Serology vs stool (%)	67	84	57
18 mo	Stool vs serology (%)	65	81	75
	Serology vs stool (%)	75	73	65
24 mo	Stool vs serology (%)	71	76	68
	Serology vs stool (%)	68	79	71
Any time point	Stool vs serology (%)	87	79	87
	Serology vs stool (%)	87	79	87

[a]n= number of children analyzed by both stool antigen test and the in-house serum IgA ELISA test.

Association between H. pylori infection and socioeconomic factors, enteric pathogens and malnutrition (Paper II)

As described by others, poor hygiene and overcrowding are factors associated with *H. pylori* infection (25, 88). Therefore, we studied different socioeconomic parameters in the Bangladeshi birth cohort children that may be related to *H. pylori* infection. In analogy with others (88), we observed that parents' literacy (both maternal and paternal) may play a significant role for *H. pylori* infection. We also found that *H. pylori*-infected children were more often infected by multiple enteropathogens, often isolated at different time points. Malnutrition did not seem to promote colonization by *H. pylori*; rather, our data indicate that wellnourished children were more susceptible to infection. However, we did not enroll severely malnourished newborns weighing less than 2 kg at birth, and can therefore not comment on the susceptibility to *H. pylori* infection in such children.

Association between H. pylori infection and ABO blood group (Paper II)

It has been reported earlier that individuals with certain ABO blood groups may be more susceptible to different enteric infections, e.g. blood group "O" subjects are more susceptible to *V. cholerae* O1 infection than persons with other blood groups (48, 57). We observed that children with blood group "A" in the birth cohort study were significantly more often infected with *H. pylori* than children with other blood groups (Table 4). When the same birth cohort children were analyzed for enterotoxigenic *Escherichia coli* (ETEC) infections, it was found that individuals having blood groups "A" and "AB" were more susceptible to ETEC (Table 4) (112). These associations between blood groups and infection with different gastrointestinal pathogens may be explained by observations that certain bacterial antigens may bind to blood group associated antigens that are expressed on epithelial cells in the small intestine (64).

Table 4. Association between ABO blood group and different gastrointestinal infections.

Blood group	*H. pylori*	ETEC	*V. cholerae* O1
A	+	+	-
B	-	-	-
AB	-	+	-
O	-	-	+

+ Indicates that individuals with a specific blood group are more susceptible to the respective infection than subjects with other blood groups.

Seasonality of onset of H. pylori infection in relation to other gastrointestinal infections

Seasonal variations of different enteric infections have been reported earlier, e.g. in Bangladesh (112) and in different countries in Africa and Latin America (118, 145). Therefore, we were interested to examine whether infection with *H. pylori* also occurred more frequently during certain periods of the year. For other gastrointestinal pathogens, peak infections are usually observed twice a year in Bangladesh. A similar trend was observed for *H. pylori* infections in the birth cohort study, i.e. most children were infected during the spring or autumn (Figure 8). The observed seasonality for *H. pylori* infection may be related to recent findings that *H. pylori* may be transmitted via vomit from patients with diarrhea (66).
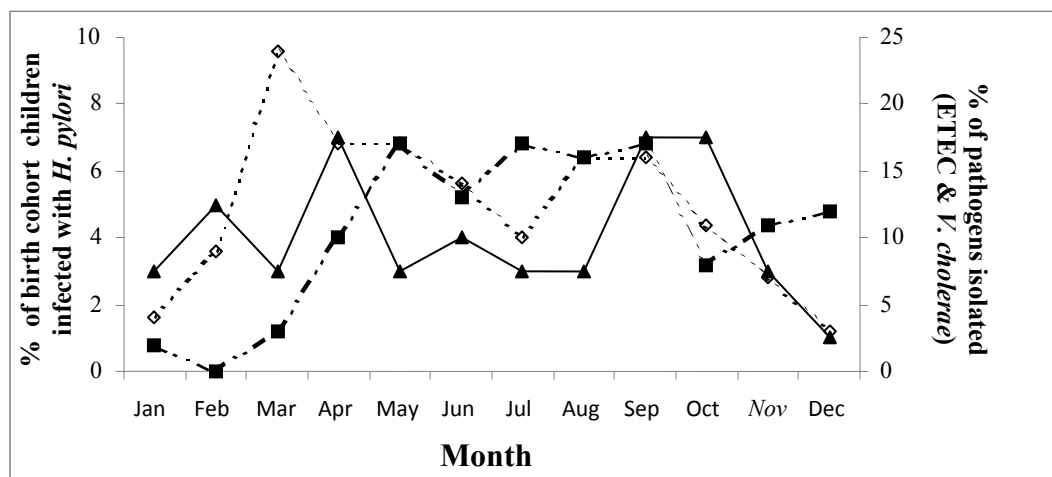

Figure 8. Seasonality of onset of *H. pylori* infection (▲) in the birth cohort compared to infection rates with ETEC (◊) and *V. cholerae* (■) in Dhaka. The ETEC and *V. cholerae* data were obtained from a 2% systemic sampling of patients with diarrhoea visiting the ICDDR,B hospital in Dhaka during 2007-2008.

Studies of the development of systemic and mucosal antibody responses to *H. pylori* infection in children (Paper III)

Studies of immune response to *H. pylori* in children during their first 2 years of life are scarce (50, 54). Since we had observed a very high incidence and prevalence of *H. pylori* infections in the birth cohort in Bangladesh (Paper II), we also studied immune responses in relation to *H. pylori* infection in these children in more details (Paper III). In initial studies, we determined serum antibody responses to *H. pylori* infection in children being infected during the first and second year of life. These analyses showed a rapid decrease in IgG titers in serum during the initial 6 months in both groups of children and also in *H.*

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pylori negative controls. Thereafter, titers increased rapidly during the remaining 2 year study period, both in children being infected during the first and second year, although children became stool antigen positive between 12 and 24 months had significantly higher IgG titers at 6 months than children being infected at ≤12 months of age. IgG titers in stool antigen negative children also increased during the study period, although to lower mean levels than the stool antigen positive children, probably due to development of antibodies cross-reacting with the *H. pylori* MP antigen used in the serological assays. The IgA antibody levels in sera of all children studied were very low before 6 months of age but increased thereafter, somewhat more rapidly in children being infected during the first year than in those being infected during the second year. The IgA antibody levels were significantly higher in the *H. pylori* stool antigen positive children after infection than corresponding levels in age matched *H. pylori* negative control children.

H. pylori specific antibody responses in spontaneously eradicated and reinfected children (Paper III)

We observed that 11% of the birth cohort children infected between 0 and 24 months of age were able to spontaneously eradicate the *H. pylori* infection. To evaluate if this eradication was associated with development of specific immune responses we compared specific antibody responses in serum and stool over time in continuously infected (CI) children with corresponding responses in spontaneously eradicated (SE) children. We also analyzed whether children who were reinfected (RI) had decreased antibody levels before reinfection. This was done by measuring serum IgA and IgG as well as stool IgA/total IgA levels in the SE, CI and RI children before and after onset of infection.

Analysis of *H. pylori* specific IgA levels in stool showed that stool IgA levels were high in most children during their first 3-6 months of life, irrespectively of infectious status, and decreased thereafter (Figure 9a-c). This pattern probably reflects contaminating breast milk antibodies in the stool samples.

Most of the SE children responded strongly in serum IgA to the infection and this response seemed to be indicative of the duration of the infection (as exemplified in Figure 9a). Substantial specific IgA/total IgA responses to the infection were also observed in stool specimens collected at ≥6 months in most of the SE children, but those responses persisted for shorter periods than the serum IgA antibody responses. Serum IgG antibody

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levels increased after infection and remained elevated also after clearance of the infection (Figure 9a).

a
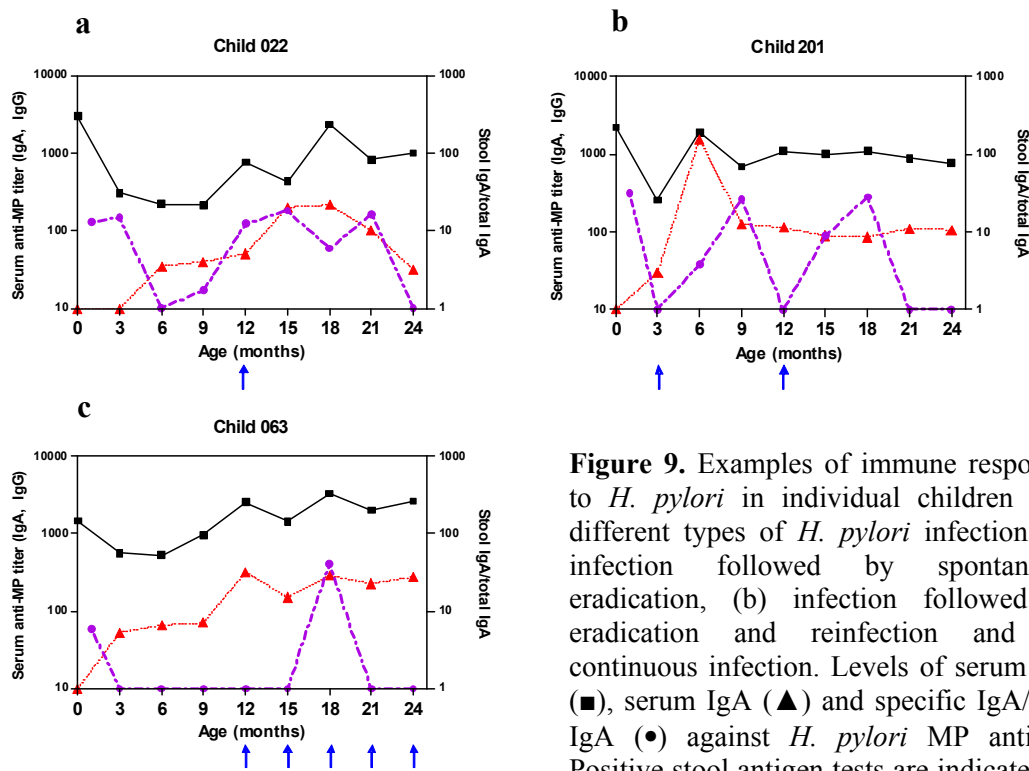Child 022

b
Child 201

c
Child 063

Figure 9. Examples of immune responses to *H. pylori* in individual children with different types of *H. pylori* infection; (a) infection followed by spontaneous eradication, (b) infection followed by eradication and reinfection and (c) continuous infection. Levels of serum IgG (■), serum IgA (▲) and specific IgA/total IgA (•) against *H. pylori* MP antigen. Positive stool antigen tests are indicated by arrows (↑).

The RI children (as exemplified in Figure 9b), showed decreased immune responses shortly before reinfection in all specimens, as compared to the SE children (figure 9a). For the RI children we did not observe any persisting levels of serum IgA or stool IgA titers after the first infection, suggesting increased susceptibility to infection, possibly due to decreased *H. pylori* specific immune responses.

In the CI children, the serum IgA and IgG levels remained very stable during the infection (as exemplified in Figure 9c). Although the stool IgA antibodies increased transiently after onset of infection, the CI children did not clear the infection (Figure 9c).

Association between antibody levels in maternal blood and breast milk and protection against acute H. pylori infection (Paper III)

We observed a very low prevalence of *H. pylori* infections in the birth cohort children during their initial 6 months of life. Based on this we explored whether transplacentally transferred IgG and/or IgA antibodies in breast milk from the mothers may be related to

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this low prevalence. We have already shown a high correlation between the IgG titers in mother's blood and in cord blood (Figure 6) that clearly suggests transplacental transfer of IgG. No IgA titers were observed in cord blood and very low levels of serum IgA were noted during the first 6 months of life. These observations, together with the drastic drop in serum IgG during the first 6 months, support a maternal origin of most of the serum antibodies during infancy.

To evaluate whether pre-existing serum IgG and/or IgA antibody levels in the children may be associated with delayed onset of infection ("protection") against *H. pylori,* we analyzed antibody titers in the serum samples collected as close as possible before children became stool antigen positive. Such analyses were undertaken both for children being infected at ≤12 months as well as for children infected between 12 and 24 months of age. IgG and IgA titers in the pre- and post-infection sera were compared with titers in sera collected at the corresponding time points from age-matched, stool antigen negative control children in the birth cohort. These analyses showed that IgG titers in preinfection sera of children being infected ≤12 months were significantly lower (P=0.02) than in sera from the *H. pylori* uninfected controls. However, no difference was observed between the levels of IgA antibodies in preinfection sera of children being infected this early or in sera from the *H. pylori* uninfected control children. Similarly, IgG and IgA titers of children being infected after 12 months of age did not differ significantly from the titers of the *H. pylori* uninfected controls. These results, showed that children with high IgG antibody levels in serum are less susceptible to *H. pylori* infection than children with low IgG titers during the first year of life, may suggest a protective role of transplacentally derived IgG antibodies.

With regard to breast milk antibodies against *H. pylori*, the levels were comparable from 1-12 months of lactation in most of the mothers. We found that children who became stool antigen positive during the first months of life drank breast milk that contained significantly lower levels of *H. pylori* specific IgA antibodies than children who became stool antigen positive at 6 months (Paper III). No significant association was observed between IgA levels in breast milk and serum antibody levels of the mothers, excluding the notion that the association between low breast milk IgA titers and early onset of infection was due to low levels of transplacentally transferred IgG antibodies.

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Thus, our results support that increased levels of IgG in cord blood and IgA antibodies in breast milk are associated with protection against early onset of *H. pylori* infection.

Analysis of cytokine and chemokine responses to *H. pylori* in Bangladeshi children and adults (Paper I & IV)

Our studies of immune responses to *H. pylori* in young children thus suggest that antibodies provided by the mother as well as antibody responses induced by the children themselves may be associated with delayed onset and/or clearance of infection. However, the antibody responses elicited by the infection may reflect a more general immune response, also involving T cells and other components of the childrens´ immune systems. Recent studies suggest that both Th1 and Th17 cells may be induced by *H. pylori* infection in adults (21, 85, 95) and that both types of T cells may contribute to vaccine induced protection in mice (32, 144). However, little is known about the importance of these types of responses in relation to *H. pylori* infection in young children. Previous studies of the cytokine profile of T cells responding to *H. pylori* have also mainly been performed in developed countries. Therefore, we analyzed the cytokine responses to *H. pylori* in both children and adults in Bangladesh.

Mucosal cytokine and chemokine responses in adults (Paper I and IV)

Analysis of gastric biopsies showed that both IL-17 and IFN-γ mRNA were expressed in the gastric mucosa of Bangladeshi as well as Swedish adults (Paper IV). The results also suggest that that the expression was induced by *H. pylori* infection, since the levels of both cytokines were higher in infected compared to uninfected individuals. IL-17 induces the production of IL-8 (72) and strong correlations have previously been found between the levels of IL-17 and IL-8 in *H. pylori* infected mucosa (95). IFN-γ, on the other hand, is a strong inducer of the chemokines IP-10 and MIG and the receptor for these cytokines, CXCR3, is mainly expressed by Th1 T cells (74). Consistent with the expression of both IL-17 and IFN-γ in the infected gastric mucosa, we found increased levels of the chemokines IL-8, IP-10 (Figure 10) and MIG (not shown) in the infected gastric mucosa of Bangladeshi volunteers, compared to uninfected mucosa of Swedish or Bangladeshi participants (Paper I).
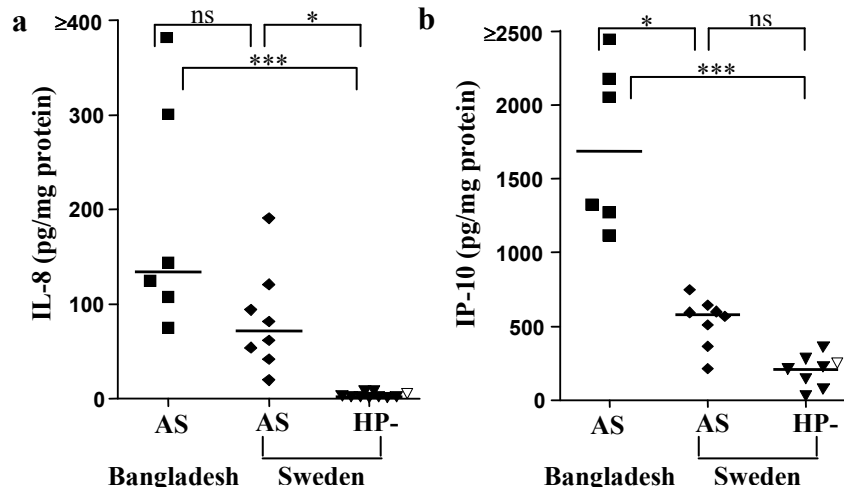
Figure 10. Levels of IL-8 (a) and IP-10 (b) protein in biopsy extracts of antral mucosa. Median values are indicated by horizontal lines. The open triangle (∇) shows the levels of chemokines in one Bangladeshi *H. pylori* uninfected control individual. ($^{*}P<0.05$, $^{***}P<0.001$; ns: not significant).

Our results also suggest that the levels of IP-10 protein as well as IFN-γ mRNA were higher in Bangladeshi compared to Swedish gastric mucosa, which may indicate a stronger Th1 response in Bangladeshi individuals. However, the mRNA results need to be confirmed in a larger number of participants, including younger Swedish subjects, since the Swedish volunteers included in the study so far were considerably older than the Bangladeshi participants. Nevertheless, our preliminary analyses of mucosal cytokines and chemokines support that both Th1 and Th17 cells contribute to the T-cell responses to *H. pylori* in Bangladeshi adults.

Cytokine responses to H. pylori in PBMCs (Paper IV)
The results from the mucosal analyses in adults encouraged us to also investigate Th17 and Th1 responses in Bangladeshi children. However, since it is almost impossible to get biopsy material from young children in Bangladesh, we instead analyzed whether T cells from young children have a general ability to respond with both IFN-γ and IL-17 production after stimulation with *H. pylori* antigens as well as polyclonal stimuli.

When PBMCs from individuals of different ages were stimulated with *H. pylori* MP, significantly higher IL-17 responses were observed in cells from infants compared to adults (Figure 11). In contrast, the IFN-γ production was comparable in the different

study groups. Very low levels of Th2 cytokines (IL-13, IL-4, IL-5) were detected in culture supernatants from any of the subjects. In contrast to the MP-induced responses, polyclonal PHA stimulation gave rise to significantly lower production of both IL-17 and IFN-γ in cells from infants compared to adults, whereas the IL-13 production was slightly higher in PBMCs from infants (Figure 11).
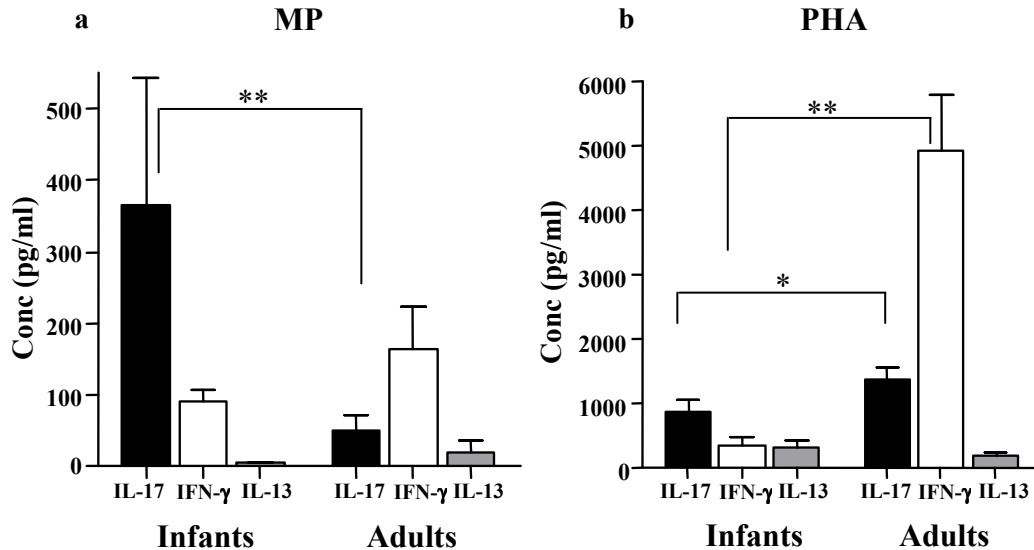

Figure 11. Production of IL-17 (black bars), IFN-γ (white bars) and IL-13 (grey bars) from PBMCs isolated from infants and adults in response to MP (a) and PHA (b) stimulation. Values shown are means + SEM from 12-16 (IL-17, IFN-γ) or 5 (IL-13) individuals. ([*]$P<0.05$, [**]$P<0.01$).

Isolated CD4^{+} T cells from infants also responded with lower production of IL-17 and IFN-γ than CD4^{+} T cells from adults when stimulated with beads coated with anti-CD3 and anti-CD28 antibodies in the absence of antigen presenting cells. Bead stimulation also induced significantly more IL-5 in infant than adult CD4^{+} T cells and there was also a tendency towards more IL-13 production in infant CD4^{+} T cells. However, despite these signs of an increased ability to produce Th2 cytokines during infancy, the T-cell responses to *H. pylori* antigens in infants were primarily of the Th17 and Th1 types.

Analysis of the origin of the cytokine responses in PBMC cultures (Paper IV)
To determine the origin of the IFN-γ and IL-17 responses observed in response to MP stimulation, we analyzed the cytokine responses in PBMCs with and without CD4^{+} T cells. Depletion of CD4^{+} T cells resulted in complete abolishment of IL-17 responses in

both infants and adults and also in reduction of IFN-γ responses in almost all individuals. These results suggest that the IL-17 responses observed in the PBMC cultures were derived from CD4$^+$ T cells, but that other cells, including CD8$^+$ T cells and NK cells, may contribute to the IFN-γ responses.

Analysis of cytokine responses in relation to infection status (Paper IV)

For the IL-17 responses, we could not detect any differences between infected and uninfected subjects. Similar IFN-γ responses were also observed in cells from infected and uninfected infants and children. In contrast, higher IFN-γ responses were observed in cultures with PBMCs isolated from infected compared to uninfected adults. The responses in uninfected individuals may be a result of previous exposure to *H. pylori* as well as cross reactivity with other bacteria expressing similar antigens. However, further studies are needed to explain the different patterns of IL-17 and IFN-γ secretion observed in the different study groups. These studies will include analysis of the influence of APC derived cytokines for the production of Th1 and Th17 cytokines in infants and adults.

Collectively, our results suggest that CD4$^+$ T cells can respond with production of both Th1 and Th17 type cytokines to *H. pylori* during childhood as well as adult life. Further studies will aim to determine the relative importance of these responses in the early stages of *H. pylori* infection and during chronic infection.

GENERAL DISCUSSION

The incidence of *H. pylori* infections has decreased substantially in the developed world during recent years (42). However, both the incidence and prevalence of such infections have been very high and remained stable in many developing countries (42, 73) and in certain countries in Eastern Europe (65) during recent decades. This is supported by the results of this thesis showing that up to 90% of adult volunteers from Dhaka in Bangladesh and/or adults living in an under-privileged area in Mirpur in Dhaka city, were positive for *H. pylori*, both as assessed by a highly specific and sensitive stool antigen test (Paper I, IV) and by serology (Paper I, III, IV). Furthermore, a very high incidence, approximately 60%, of *H. pylori* infections was observed in children already during their initial two years of life in the Mirpur area using the same stool antigen test and serology (Paper II). This finding confirms results from other studies showing that the majority of *H. pylori* infections occur during early childhood (25, 88).

Most studies of immune responses to *H. pylori* have been undertaken in chronically infected adults living in developed countries in the world (134). However, very few studies have analyzed immune responses to *H. pylori* infection in highly endemic areas in developing countries, or immune responses to acute *H. pylori* infections. To determine how well results from studies in low endemic countries may reflect those from individuals living in high endemic areas we undertook a detailed comparison of immune responses in Bangladeshi and Swedish *H. pylori* infected subjects (Paper I). Thus, we hypothesized that individuals living in areas with an increased incidence of enteric infections and parasitic infestations as well as other confounding factors such as calorie and micronutrient deficiencies might respond less well immunologically to *H. pylori* infection (122). However, our analyses showed that mucosal B- as well as T- cell responses to *H. pylori* in the stomach in the Swedish and Bangladeshi *H. pylori* infected individuals were very similar and suggest that findings of mucosal immune responses to *H. pylori* infection in West European and American subjects may be applicable also to individuals living in the developing world. Even the numbers of FOXP3$^+$ Treg were comparable in stomach as well as duodenal mucosa of Bangladeshi and Swedish adults, suggesting that the immunological hyporesponsiveness often observed in individuals in developing countries may not be a direct result of increased Treg suppression induced by

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recurrent infections. However, our finding of about 2-fold lower mean serum antibody titers to *H. pylori* in the Bangladeshi than in the Swedish age-matched subjects may be important both with regard to a putative protective role of serum antibodies (see below) as well as for determining cut off levels for antibody titers signifying seropositivity. Thus, cut off levels for seropositivity may differ in different populations and should be recognized before use in a certain setting based on comparison with other diagnostic tools, e.g. culture, UBT or stool antigen tests (23, 28, 31).

Determination of immune responses against acute *H. pylori* infections may be undertaken in human volunteers challenged with virulent *H. pylori* bacteria. Such studies may reveal the kinetics of immune responses at various time points after infection. A few such challenge studies have been undertaken (53, 102), but these have been performed in adults and not in children who are more likely to acquire *H. pylori* infection and who may respond differently to initial infection compared to adults. Another possibility to study immune responses against acute *H. pylori* infections is to collect clinical samples from young children in areas with a high incidence of *H. pylori* infections and relate development of immune responses to onset of infection as determined by e.g. UBT or positive stool antigen test. We have chosen the latter approach and collected blood and stool samples regularly during the first two years of life from a birth cohort of children with a very high incidence of *H. pylori* infections (Paper II) and determined antibody responses in relation to onset of infection (Paper II, III). This has included assessment of IgA and IgG antibody responses in serum as well IgA/total IgA rises in stool following onset of *H. pylori* infections during the first two years of life. We have also studied T-cell responses in infants and young children in the same setting in *H. pylori* positive and negative children (Paper IV). However, since T-cell responses are more technically demanding to analyze than antibody responses, we limited our T-cell studies to children with and without *H. pylori* infection at a given time point and, in contrast to the antibody analyses, did not attempt to follow the development of T-cell responses to *H. pylori* over time.

We detected at least fourfold rises in IgG and IgA titers to *H. pylori* in serum in a majority of the children during the two year study period. The frequency of seropositive children in the birth cohort was also considerably higher than in other studies of immune

responses of *H. pylori* in young children in developed and developing countries (51). The high seropositivity may be due to the unusually high incidence of infections in the birth cohort. However, it can also be due to the fact that we used a very sensitive in-house ELISA test and assessed end point titers (92), at variance with other studies which have utilized commercial ELISA tests which are based on analysis of single dilutions of clinical specimens (75, 82).

Due to the very high frequency of *H. pylori* positive adults in Bangladesh, almost all the children in the birth cohort had very high IgG titers in serum at birth due to transplacentally transferred antibodies from the mothers (Paper III). These titers remained for at least 6 months after delivery, making it impossible to determine IgG responses to *H. pylori* infection in serum in the children during their first 6 months of life. Thereafter, antibody titers to *H. pylori* increased with age in relation to onset of infection but also, although of significantly lower magnitudes, in stool antigen negative children. IgA titers were negligible or very low in all children during the first 3-6 months of life but increased thereafter in response to infection, but also to some extent in stool antigen negative children. Since we used a whole membrane protein antigen preparation of *H. pylori* bacteria as coating antigen in the ELISA, the titer rises observed in the stool antigen negative children may be due to infections with other bacteria expressing cross-reactive protein antigens. These titer rises may also be explained by infection with low levels of *H. pylori* that could not be detected by the stool antigen test used, or by transient infection with *H. pylori* that could not be detected by our 3 or 6 monthly stool antigen analyses (Paper II, III). Our finding of maternal IgG titers in serum during infancy and the observation that such titers remained for considerably longer periods than corresponding serum IgA titers after spontaneous eradication of infection, support that determination of systemic antibody responses to *H. pylori* in young children should preferably be based on IgA antibody analyses.

We also explored the possibility of determining mucosal antibody responses to *H. pylori* in stool extracts, using the same approach as we have successfully applied for determining mucosal antibody response to oral enteric vaccines (5) (Paper III). However, we found that most stool samples had very high IgA titers against *H. pylori* during the first 6 months of life, irrespective of if the children were infected or not. Such anti-*H.*

pylori antibodies in stool probably reflect "contamination" with breast milk antibodies, since all children were almost exclusively breastfed during the initial 6 months of life, and most mothers had high levels of anti-*H. pylori* IgA titers in milk during the entire lactation period. During the remaining study period between 6 and 24 months, on the other hand, specific IgA/total IgA rose at least 4-fold in stool after infection in most children, but these responses were considerably more short-lasting than the systemic responses (Paper III) as also observed for mucosal immune responses to other enteric infections (113, 133).

In analogy with a previous study, we found a relatively high incidence of spontaneous eradication of *H. pylori* infection in the young age group of children studied (93) (Paper II, III). These spontaneous eradications seem to be related to development of specific immune responses against *H. pylori* infection (Paper III). Thus, we observed substantial titer rises both in serum and in stool in relation to onset of the infection in the eradicated children; these responses were considerably higher than corresponding responses in continuously infected children. However, it is not possible to say whether the antibody responses in the eradicated children were protective *per se* or only reflected protection. Studies in different animal models have indicated that Th1 as well as Th17 immune responses are crucial for protection against *H. pylori* infection (32, 105, 144), but their role in protection from *H. pylori* infection in humans needs to be further studied. Support for a protective role of specific anti-*H. pylori* antibodies, on the other hand, is our findings that children infected with *H. pylori* during the first year of life had significant lower levels of specific IgG antibodies in serum than children who became infected during the second year. Furthermore, children who drank milk with low levels of anti-*H. pylori* IgA antibodies were infected with *H. pylori* considerably earlier than children fed breast milk with higher levels of specific IgA titers (Paper III). A relation between specific breast milk antibody titers against urease and protection against colonization by *H. pylori* has been observed in Gambian infants (138). Furthermore, a recent meta-analysis has supported that breast feeding is protective against *H. pylori* infection, with an effect primarily seen in studies in LMIC (24).

In addition to a protective role of specific antibodies against acute *H. pylori* infection during early childhood, our results suggest that Th17 as well as Th1 type T- cell

responses may contribute to effective immune responses against *H. pylori* (Paper IV). Thus, we observed that CD4$^+$ T cells from infants were able to produce relatively high levels of both IL-17 and IFN-γ in response to stimulation with *H. pylori* antigens. These findings are interesting in relation to the recent demonstration of the importance of IL-17 producing cells for the recruitment of neutrophils to the gastric mucosa in immunized and challenged mice (32). In immunized mice, neutrophil depletion resulted in increased bacterial colonization of the gastric mucosa after challenge (32), suggesting that Th17 cells can play a role in limiting the infection. Interestingly, several recent studies also suggest that Th17 cells can promote B-cell survival and differentiation (33), enhance B-cell recruitment to mucosal sites (62) and increase the expression of the polymeric immunoglobulin receptor on epithelial cells, thereby elevating secretory IgA levels in mucosal secretions (63). However, whether both antibodies and Th17 cells may contribute to the delayed onset and spontaneous eradication of *H. pylori* infection observed in children in the birth cohort remains to be further investigated.

In this thesis, we have also evaluated different socioeconomic factors that may predispose to *H. pylori* infection during early childhood (Paper II). In accordance with several other studies we confirm that low maternal (12, 88), and in this thesis also low paternal education level (Paper II) were associated with increased susceptibility to *H. pylori* infection in the birth cohort in Mirpur. However, other socioeconomic factors such as access to clean water, latrines, family crowding etc did not relate to incidence of infection. Interestingly, we observed a clear seasonality with regard to onset of infection that seemed to relate to peak seasons for bacterial enteric infections (Paper I). To our knowledge, such a seasonality of *H. pylori* infections has not been observed previously. The seasonality may be explained by our recent finding of high numbers of transcriptionally active *H. pylori* bacteria in vomitus from individuals with diarrhoeal diseases (66). Thus, in crowded areas with poor hygiene vomiting caused by gastrointestinal infections may promote the transmission of *H. pylori*. We also found that children with blood group "A" had a significantly increased susceptibility to *H. pylori* infection compared to children with other blood groups, which is at variance with another report in which no relation between ABO blood group and susceptibility to *H. pylori* infection was observed (120). A similar relationship between blood group "A" and ETEC

infection was observed in the same birth cohort as studied in this thesis (112). The reason for this increased susceptibility in blood group "A" children to two such different mucosal pathogens is unclear at present, but will be explored in future studies of binding structures of *H. pylori* and ETEC colonizing factors (64).

In conclusion, this thesis describes a number of different factors that may promote as well as prevent *H. pylori* infection during early childhood. Our findings may play a role in the development of effective preventive and treatment strategies that should be particularly important for those who are at highest risk of being infected with *H. pylori*, e.g. children in high endemic countries, especially children in the developing world that have less opportunities for conventional antibiotic therapy regimens (4, 134).

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