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HUMORAL IMMUNE RESPONSE TO *HELICOBACTER PYLORI*: A STUDY OF HOST-DEPENDENT AND MICROBIAL FACTORS

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Tellimus nr. 620

To my little daughter Katarina
for giving me
the will, energy and faith

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred to in the text by their Roman numerals:

I. **Klaamas K**, Held M, Wadström T, Lipping A, Kurtenkov O. IgG immune response to *Helicobacter pylori* antigens in patients with gastric cancer as defined by ELISA and immunoiblotting. Int J Cancer 1996; 67: 1–5.

II. **Klaamas K**, Kurtenkov O, Ellamaa M, Wadström T. The *Helicobacter pylori* seroprevalence in blood donors related to Lewis (a,b) histo-blood group phenotype. Eur J Gastroenterol Hepatol 1997; 9: 367–370.

III. Kurtenkov O, **Klaamas K**, Miljukhina L, Shljapnikova L, Ellamaa M, Bovin N, Wadström T. IgG antibodies to Lewis type 2 antigens in serum of *H.pylori*-infected and noninfected blood donors of different Lewis(a,b) blood-group phenotype. FEMS Immunol Med Microbiol 1999; 24: 227–232.

IV. **Klaamas K**, Kurtenkov O, Covacci A, Lipping A, Wadström T. Immune response to a recombinant fragment of the CagA protein of *Helicobacter pylori* in blood donors and patients with gastric cancer: relation to ABO(H) blood group phenotype, stage of the disease and tumor morphology. Med Microbiol Immunol 1999; 187: 227–232.

V. **Klaamas K,** Brjalin V, Shljapnikova L, Lipping A, Kurtenkov O. *Helicobacter pylori* ja CagA seroloogiline staatus gastroduodenaalse patoloogiaga haigetel: seos peremeesorganismi ABO(H), Lewis fenotüübi ja sekretoorse (Se/se) staatusega.[*Helicobacter pylori* and CagA serologic status in patients with gastroduodenal pathology: relation to ABO(H), Lewis (a,b) phenotype and Se/se status of the host]. Eesti Arst 2003; 82: 249–255.

VI. **Klaamas K**, Kurtenkov O, Brjalin V, Miljukhina L, Shljapnikova L, Engstrand L. Enhanced humoral immune response to tumor-associated T glycotope (Galβ1,3-GalNAc) in *Helicobacter pylori*-infected blood donors, patients with gastric cancer and benign gastric conditions. Exp Oncol 2002; 24: 38–44.

VII. **Klaamas K**, Kurtenkov O, Rittenhouse-Olson K, Brjalin V, Miljukhina L, Shljapnikova L & Engstrand L. Expression of tumor-associated Thomsen-Friedenreich antigen (T Ag) in *Helicobacter pylori* and modulation of T Ag specific immune response in infected individuals. Immunol Investigations 2002; 31: 191–204.

VIII. Kurtenkov O, **Klaamas K**, Sergeyev B, Chuzmarov V, Miljukhina L, Shljapnikova L. Better survival of *Helicobacter pylori* infected patients with early gastric cancer is related to a higher level of Thomsen-Friedenreich antigen-specific antibodies. Immunol Investigations 2003; 32: 83–93.

ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BabA	*H.pylori* adhesin for Le(b) antigen
BSA	bovine serum albumine
cagA	cytotoxin-associated gene A
CagA	cytotoxin-associated CagA protein, coded by *cagA*
ELISA	enzyme-linked immunosorbent assay
H.pylori	*Helicobacter pylori*
HSP60	heat shock protein 60 kDa
IgA,G,M	immunoglobulin class A, G, M
IL	interleukin
IFN	interferon
kD	kilodalton
Le(a,b,x,y)	Lewis antigens a,b,x,y
LPS	lipopolysaccharide
MAb	monoclonal antibody
NAP	neutrophil activating protein
NBT	nitro blue tetrazolium
O.D.	optical density
PAI	pathogenicity island
PBS	phosphate buffered saline
RAA	relative antibody activity
SabA	sialic acid binding adhesin of *H.pylori* (a receptor for sialyl-dimeric-Lewis x glycosphingolipid)
SDS-PAGE	sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
Se/se	secretory status: secretor/non-secretor
TNFα	tumor necrosis factor alpha
T antigen	Thomsen-Friedenreich antigen (Galβ1,3GalNAc)
TRIS	tris(hydroxymethyl) aminomethane
vacA	a gene coding for vacuolating cytotoxin
VacA	vacuolating cytotoxin

INTRODUCTION

Helicobacter pylori is a spiral microaerophilic, Gram-negative bacterium 2–5 μm long, 0.5–1μm in diameter, and with 1–6 unipolar flagella (Marshall and Warren, 1983). *H.pylori* colonizes under a mucous layer on the surface of the epithelium of human gastric mucosa. Bacteria adhere to the gastric epithelial cells and trigger intracellular signalling pathways leading to the damage of the host cells (Haas et al., 2001; Stein et al., 2002). The recognition that *H.pylori* is almost invariably associated with long-term chronic active gastritis, responsible for peptic ulcer, increased risk for gastric cancer and MALT lymphoma is the most important advance that has been made in gastroenterology over the past decades (Axon, 1999). This issue is currently one of the most active research topics in biomedicine. The complete genome of two *H.pylori* strains is now sequenced (Alm et al., 1999). This information will allow for the selection of novel target gene candidates that are closely related to the virulence of *H.pylori* and the pathogenesis of *H.pylori*-associated diseases.

Although *H.pylori* is believed to infect more than half the world's population, marked differences in prevalence exist between different countries and geographical areas. Approximately 40 and 80 percent of individuals in developed and developing countries, respectively, are infected. In Estonia, more than 80% of the adults are infected, and the incidence of *H.pylori*-associated diseases, including gastric cancer, is also extremely prevalent (Maaroos et al., 1990; 1995; Vorobjova et al., 1994, 1998; Thomson et al., 1996).

If not treated an infection by *H. pylory* persists for decades. However, a majority of infected remain asymptomatic and never develop overt disease or symptoms. In 10–20% of carriers, gastroduodenal disease develops including gastric or duodenal ulcer, type B chronic and atrophic gastritis, which is a precondition to gastric cancer (Siurala et al., 1988; Kuipers et al., 1995b; Correa and Miller, 1998; Sipponen, 2002). However, the precise mechanisms of mucosal injury, ulceration and gastric carcinogenesis are not fully understood. This is due to no reliable indicators which predict the clinical outcome of the infection at the individual level. Therefore, it is a big challenge to determine why the disease strikes only a small percentage of *H.pylori* carriers and what determines the differences in the clinical outcome of the infection. At least three main possibilities have been considered: (i) *H.pylori* diversity and the existence of more virulent strains or the strains which are preferably involved in the pathogenesis of a particular disease; (ii) unique or aberrant host response to infection contributes to a specific outcome; (iii) other co-factors, such as enviromental exposures, that can modulate the *H.pylori*-host interrelationships. It seems, however, that the right combination of several factors in a genetically susceptible host determines a specific clinical outcome. Many evidences support these considerations. (Blaser et al., 1995,1996; Finlay and Fialkow,

1997; Dubois et al., 1999; Casadevall & Pirofski, 2000; Blaser, 2000). However, it should be *a priori* expected, that an interplay between two such genetically diverse, polymorphic systems, such as *H.pylori* and the host could not lead to a single effect.

On the basis of the evidence to date, the *H.pylori* infection is a typical 'slow infection' where the host is the major factor in determining the natural course and clinical outcome of the infection (Nguen et al., 1999; Blaser, 2000, 2002). To evaluate the role of host polymorphism in the host-*H.pylori* immunologic interplay we monitored an immunologic response of the individual to *H.pylori* in relation to a highly polymorphic system of ABO(H) and Lewis blood group antigens which were used as predictors.

H.pylori diversity at genetic and phenotypic level

Analysis of the *H.pylori* genome, predicts a small genome (~1.6–1,7Mb) with relatively few regulatory genes needed for adaptation to the environment (Tomb et al., 1997). The first genomic comparison of two unrelated *H.pylori* isolates showed that about 40% of the genes were of unknown function and only 6 to 7% of the genes were specific to each strain, with almost half of them being clustered in a single hyper-variable region (Alm et al., 1999). Salama et al. (2000) found that out of 1643 genes analyzed in 15 strains of *H.pylori*, 1281 were common to all strains suggesting that about 20% of the genes may be strain specific. Different human populations also show appreciable differences in genotypes of *H.pylori* (Kersulyte et al., 2000).

Many important virulence genes of pathogenic bacteria are often grouped in genetic elements termed pathogenecity islands (PAIs) (Hacker et al.,1997). More than half of the *H.pylori* strains contain a pathogenecity island (cagPAI), termed the cag region, whose presence has a marked influence on the virulence of the organism (Censini et al., 1996). The cag region was probably acquired by DNA uptake from a different species (Censini et al., 1996). The cagPAI encodes for the CagA (cytotoxin associated protein), which may be translocated into host cell cytoplasm through a type IV secretion machinery, phosphorylated by the host cellular kinases, and converted into tyrosine-phosphorylated CagA. (Odenbreit et al., 2000; Haas et al., 2001; Censini et al., 2001). The molecular mass of CagA varies from 120 to 145 kDa among the strains due to variability in the cagA 3' region. This may alter tyrosine phosphorylation sites and the duplication of a varied number of a 102-bp repeat sequences (Covacci and Rappuoli, 1998; Dong et al., 2002). It has now been suggested that the cag sequence is the major player in the host-pathogen relationships (Censini et al., 2001).

The unusually high degree of diversity of *H.pylori* strains suggests that natural transformation may occur *in situ*. Recombination between *H.pylori* strains is extremely common (Sauerbaum et al., 1998) and novel subtypes appear during colonization (Owen et al., 1994; Kersulyte et al., 1999). Colonization with two or more strains is typical. (Taylor et al.,1995; Figura et al.,1998). Genetic analysis showed mosaicism for many genes, in particular vacuolating cytotoxin (*vacA)* and *cagA* genes (Cover et al.,1994; Atherton et al.,1995; Logan and Berg, 1996; Dong et al., 2002). Similar polymorphism has been shown for some other genes of *H.pylori* such as *ureA*, *iceA* (induced by contact with epithelium) (Peek et al., 1998, 2000) and phospholipase A (*pldA*) gene (Xerry and Owen, 2001), however, their association with pathogenicity has not been determined. There is also substantial evidence that some genes may be expressed only within the gastric environment or activated upon contact with the host cells (*iceA*) or in response to a 'unique' cellular signature (*ureA, nap)* (Finlay and Falkow, 1997; Blom et al., 2002).

The pathogenecity of microbes is closely related to their phenotypic diversity which allows their adaptation to host microenvironments and to evade the host immune response (Deitsch et al., 1997). Several phenotypic characteristics are known to vary among *H.pylori* strains. These include the structure of lipopolysaccharides (LPS), the production of the *cagA*-encoded protein (CagA, 120–140 kDa), expression of vacuolating cytotoxin (VacA), Cover et al., 1994; Censini et al., 1996), BabA adhesin (Moran, 1995; Gerhard et al., 1999; Printz et al., 2001), the ability to activate neutrophils (Evans et al., 1995; Satin et al, 2000), and an expression of Lewis antigens (Appelmelk et al., 1996, 1998; Wirth et al., 1999; Dundon et al., 2001; Webb and Blaser, 2002).

The significance of the LPS diversity is not yet well understood, but potentially relates to the organism's virulence and its interaction with neutrophils. LPS of *H.pylori* has low biological activity, a property which may aid in the persistance of the infection, possibly due to an antigenic mimicry mechanism: because the O-specific chain of *H.pylori* LPS mimics structurally Lewis blood group antigens in structure (Appelmelk et al., 1996; Moran, 1996). Besides, immune cross-reactivity between the bacteria and the gastric mucosa may play role in an induction of autoimmunity via antigenic mimicry mechanism (Negrini et al., 1991, 1997).

The characterization of proteins of *H.pylori* via proteomics technology including two-dimensional gel electrophoresis, showed even greater polymorphism. Three *H.pylori* strains were analyzed and more than 1500 protein spots were compared. (Jungblut et al., 2000; Nilsson et al.,2000; Alm et al., 2000). There is evidence that a single gene may create multiple proteins as a result of post-translational modification. In addition, some disease-type specific patterns were detected suggesting the ability of the approach to select candidate indicators for clinical manifestations (Haas et al., 2002).

The majority of *H.pylori* strains was shown to express human blood group related antigens: Le type I (Le a,b) and Lewis type II (Le x,y), ABH antigens

(Sherburne & Taylor, 1995; Appelmelk et al., 1996; Simmons-Smith et al., 1996; Heneghan et al., 2001). An antigenic variation depending on environmental conditions has been shown to be characteristic of these antigens (Appelmelk et al., 1998). An expression of Lewis antigens was shown to be dependent on the Lewis phenotype of the host (Wirth et al., 1997) though this issue remains contradictory (Taylor et al., 1998). At the same time, phenotypic diversity in Lewis expression of *H.pylori* isolated from the same host has been demonstrated, suggesting the continuous selection of optimally host-adapted populations suitable for persistence (Wirth et al., 1999; Blaser, 2000). It appears that there is host specificity in determining strain selection (Dubois et al.,1999).

It has been hypothesized that an enormous diversity of *H.pylori* is an indication of the long duration history of human-*H.pylori* interaction (Blaser 1996, 2000). It may reflect selection for a variety of human phenotypic characteristics that are related to the polymorphism of human population and its environment, including diet. (Blaser,1996; Webb & Blaser, 2002). Another speculative explanation might be that this diversity itself may in part account for the lifelong chronicity of infection.

Virulence factors of *H.pylori* and their clinical relevance

To date, a number of virulence factors that are known to be involved in the pathogenesis of the infection and associated with severe clinical outcome have been identified and characterized from the *H.pylori*. These are: the cag pathogenicity island (*cag* PAI), the vacuolating cytotoxin (VacA), cytotoxin-associated protein CagA, urease, the *H.pylori* neutrophil-activating protein (HP-NAP) (Satin et al., 2000), blood group Lewis(b)-binding adhesin (BabA) (Boren et al., 1993; Prinz et al., 2001), sialylated Le(x) binding adhesin (SabA) (Mahdavi et al., 2002), and some others.

The *cagA* and *vacA* genes and their products are two of the most studied virulence factors of *H.pylori*. The *cagA* is the nonconserved gene of *H.pylori* which is present in 60–90% of *H.pylori* strains and it is a marker for the presence of the cag pathogenicity island. The *cag* region is related to the phenotypic differences between type I proinflammatory (*cagA+vacA+*) and type II (*cagA-vacA-*) *H.pylori* strains (Xiang et al., 1995; Censini et al., 1996). The CagA protein is involved in downregulation of PHA-induced proliferation of T cells and their growth (Paziak-Domanska et al., 2000). The cagPAI is also associated with an increased production of proinflammatory interleukin-8 in gastric epithelial cells and increased IL-10 and IL-12 mRNA expression (Crabtree et al., 1994a,b; Hida et al., 1999).

There are appreciable geographic differences in the distribution of CagA positive strains as well as in the degree of the association with gastric diseases

(van Doorn et al., 2000). More than 90% of *H.pylori* strains isolated from Asian countries are CagA positive, irrespective of the clinical expression outcome (Maeda et al., 1997,1998). Despite some differences in the prevalence, rather similar results were observed in many geographical areas with respect to the relation between *H.pylori* genotypes and histopathology. Many reports have shown that distinct *H.pylori* genotypes are associated with histopathological findings in the stomach, confirming their relevance for the development of *H.pylori*-associated pathology. Infection with CagA positive strains is associated with an increased risk for development of peptic ulcer disease, atrophic gastritis, gastric cancer and intestinal metaplasia (Kuipers et al.,1995 *J NCI;* Kuipers 1995a; Blaser et al., 1995; Parsonnet et al., 1997; Orsini et al., 1998; Maaroos et al.,1999; Haruma et al., 2000; Asaka et al., 2001). Strains with mutations in *cag* genes showed a reduced capacity to initiate colonization in the mouse model of infection compared to wild-type strain (Marchetti and Rappuoli, 2002). However, in another experimental study performed in piglets and mice it was shown that neither the cagPAI, nor the ability to induce IL-8 *in vitro* is essential for colonization or neutrophilic inflammation and there was no direct relationships between the presence of cag PAI, IL-8 induction and neutrophilic gastritis (Eaton et al., 2001). Some authors were unable to demonstrate that cagA, vacA or other genotypes or the mosaicism of these genes might predict clinical outcomes of the infection (Go et al., 1998;; Anderson et al., 2002). The latest study of Twisk et al. (2001) found that 716 consecutive patients showed no relation of CagA status to *H.pylori* topography and colonisation density. These authors also found no correlation between anti-CagA antibody titers and above mentioned parameters. No higher risk elevation for developing of peptic ulcer disease or cancer was found in China, Korea and Japan (Park et al., 1998; Graham and Yamaoka, 2000; Goh et al., 1998). In different human populations from five continents several different types of changes were found in cagPAI (Kersulyte et al., 2000). Maeda et al., (1998) did not find any difference in CagA seroprevalence among *H.pylori* positive patients with different gastric diseases as well as no relation to the mucosa histology. Most *H.pylori* isolates were positive for VacA and CagA irrespective of pathology (Maeda et al., 1998). A recent international study (Webb et al., 1999) showed that the variation in the CagA seroprevalence did not explain geographic variation in gastric cancer rates any better than *H.pylori* seroprevalence alone. In addition, it has been shown that not all CagA proteins are structured equally and in their potential for initiating host cell responses *via* signal transduction pathway (Evans and Evans, 2001).

Thus, this issue remains controversial in part because of many patients are infected with multiple strains of *H.pylori*. A high degree of heterogenecity in many biopsies from an individual patient was detected by ribotyping, restriction enzyme and other analysis (Hazell et al., 1996). From 20 to 76% of patients were infected with two or more strains (Figura et al., 1998). However, one

strain is usually dominant in a given host. Most patients (68%) with nonulcer dyspepsia were infected by both CagA- and CagA+ *H.pylori* strains.

In 1988, Leunk and colleagues described the ability of *H.pylori* to induce vacuolization in epithelial cells. The *vacA* gene seems to be invariably present in all *H.pylori* strains. However, only some of them produce an active toxin. The *vacA* alleles of two variable regions (s and m) differ by a degree of cytotoxin production and its ability to interact with the target cells (Atherton et al., 1995; Pagliaccia et al., 1998). A maximum of cytotoxic activity was found with s1m1 allele, while no cytotoxic activity was found when the s2m2 allele was present (Atherton et al., 1995). In a mouse model, only the VacA-producing strains induced gastric lesions (Ghiara et al., 1995). Nearly all VacA+ strains are *cagA* positive. However, the *cagA* gene does not actually encode the vacuolating toxin, it is often co-expressed with VacA (Xiang et al., 1995). Two known variants of *cagA* gene *cagA1* and *cagA2* were shown to be associated with *vacA* subtypes, *vacAm1* and *vacAs1c*, respectively. The *vacAs1*, *cagA+* genotype was significantly associated with a higher *H.pylori* density, higher symptom scores in patients with functional dyspepsia (Loffeld et al., 2001), higher degrees of lymphocytic and neutrophilic infiltrates, atrophy, the type of intestinal metaplasia, and the presence of epithelial damage (Nogueira et al., 2001). CagA-positive, VacA producing strains cause significantlly stronger inhibition of gastric mucin expression or secretion (Muc5AC and Muc1) in mucin-producing cultured gastric epithelial cells (KATO III) after contact with *H.pylori* (Beil et al., 2000).

The neutrophil-activating protein of *H.pylori* (HP-NAP) is a high molecular mass immunogenic protein. It is chemotactic for leukocytes and induces the production of oxygen radicals in human neutrophils *via* a cascade of intracellular events which may contribute to the damage of the stomach mucosa (Evans et al.,1995; Satin et al 2000, Dundon et al. 2001). This stimulating effect is strongly potentiated by TNFα and IFNγ (Satin et al., 2000). A *H.pylori* mutant lacking HP-NAP was significantly less active than the wild-type strain (Montemurro et al., 2001). In addition, a low molecular weight neutrophil activating factor (<3kDa) has been demonstrated in *H.pylori* cell-free extracts (Leaky et al., 2001).

H.pylori synthesizes urease at a higher level than any other known organism (Scott et al., 1998). Urease hydrolyses urea to form ammonia and carbon dioxide which can absorb acid to form ammonium. There are seven genes in urease gene cluster of *H.pylori* (Weeks et al., 2000). The *ureI* sequence encoding the Ure I membrane protein is specific for *H.pylori* and required for acidic activation of cytoplasmic urease. Urease seems important for living in acidic environment of the stomach because *ureI*-negative mutants of *H.pylori* are not able to colonize the stomach (Tsuda et al., 1994). The *H.pylori* urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production (Harris et al., 1996). *In vitro* studies have also shown it to be toxic to human epithelial cells (Smoot et al., 1990). Urease has a dose-

dependent, acid-mediated adherence to polysachharides and gastric mucin, thus promoting colonization (Icatlo et al., 2000).

Adherence of *H.pylori* to gastric epithelial cells might be an important mechanism for virulence. This may be mediated by the blood group antigen binding adhesin BabA targeting human Lewis (b) surface glycoconjugate epitopes (Boren et al., 1993; Gerhard et al., 1999; Prinz et al., 2001). Adherence *via* BabA appears to be of importance for efficient delivery of VacA and CagA to the host epithelial cells and facilitates bacterial colonization (Printz et al., 2001; Rad et al., 2002). Another, sialic acid-binding adhesin (SabA) to sialyl-Le(x) has been recently identified by the Boren group (Mahdavi et al., 2002). The expression of sialyl-dimeric-Lewis(x) glycosphingolipid receptors in gastric epithelium was shown to increase during chronic inflammation thus promoting the chronicity of *H.pylori* infection.

Several other ligand-receptor interactions between *H.pylori* and gastric epithelial cells, including N-sialic acid-binding haemagglutinin (Lelwala-Guruge et al., 1992) and laminin-binding protein (Trust et al., 1991; Moran et al., 1993; Wadström et al.,1996), suggest that the adhesion of *H.pylori* might be a multi-factorial and multi-step process. *In vitro H.pylori* strains bind extracellular matrix components such as laminin, fibronectin, collagens and heparan sulphate (Wadström et al., 1996, Wadström and Ljungh., 1999).

Thus, *H.pylori* may use various strategies in different hosts to colonize the stomach using different targets such as proteins and gastric glycoconjugates. It is clear, however, that the carbohydrates of both *H.pylori* (LPS and other glycoconjugates) and the host play an important role in the adhesion process. It should be expected that variable expression of specific adhesins on bacterial strains as well as host polymorphism in expression of related receptors may appreciably influence this interaction.

The heat shock protein 60 (HSP60) of *H.pylori* cross-react with HSP60 of other bacteria and human HSPs. Strong immune response was demonstrated to these proteins in *H.pylori* infected subjects. (Engstrand et al., 1991, 1993). Yamaguchi et al., (2000) reported that *H.pylori* HSP also share a unique epitope which was protective against *H.pylori* infection in mice immunized with this peptide. Interestingly, the *H.pylori* infected animals had a significantly lower antibody level to this epitope than uninfected ones.

Heat shock protein B (HspB) of *H.pylori* is highly conserved. HspB gene has been cloned and sequenced (Sauerbaum et al., 1994). The HspA is unique in that it contains a nickel-binding site and may play a role in the integration of nickel into functional urease molecule. The expression of HSP60 on *H.pylori* surface was shown to correlate with the adhesion to primary human gastric epithelial and cultured gastric carcinoma cells (Yamaguchi et al., 1996, 1997). An inflammation induced by *H.pylori* leads to an enhanced expression of HSP in human gastric epithelial cells thus allowing *H.pylori* to persist.

The unique feature of the *H.pylori* LPS is its low proinflammatory activity (Moran, 1996). Low immunological response to *H.pylori* LPS may be a factor

leading to a chronic 'slow' infection. An inhibitory effect of *H.pylori* LPS on the process of mucus glycosylation and sulphatation (Slomiany et al., 1992) suggests that the appearance of carbohydrate precursors may be expected in gastric glycoconjugates during *H.pylori*-host interplay. In rats, *H.pylori* LPS can cause acute gastritis and an induction of gastric epithelial cell apoptosis (Piotrowski et al., 1997).

Other factors such as bacterial motility, the chemokine CheY1 and CheA system regulating chemotactic response, MUC5AC gastric mucin expression has been shown to be also important in *H.pylori* colonization (Foynes et al., 2000; Van den Brink et al.,2001).

Altogether, *H.pylori* possesses several virulence factors that are related to the pathogenesis of the infection. However the virulence factor model alone can not explain clinical polymorphism of *H.pylori* associates pathology (Graham and Yamaoka, 2000). No strong disease type-specific associations were demonstrated possibly because in most situations infectious diseases are not the consequence of a single virulence determinant. It appears that virulence factors of *H.pylori* are just markers for enhanced inflammation and are not directly involved in the pathogenesis of a specific gastric disease. There is now a growing evidence to suggest that virulence of *H.pylori* is largely host-dependent (Dubois et al., 1999; Graham and Yamaoka, 2000;). Variability in host immune responses may contribute to mucosal damage in *H.pylori* associated gastritis (Shimoyama and Crabtree, 1998). It appears that the inflammatory activity in *H.pylori* infection is also predominantly organism related (Michetti, 2000; Ernst and Gold, 2000). An expression of iceA gene alleles (Peek et al., 1998; van Doorn et al., 1998; Nishiya et al., 2000) is a good example. Since host factors seem to be important in determining which strain of *H.pylori* predominates, in animal models at least (Dubois et al., 1999), this may also be important in human beings for susceptibility to initial or persistent infection and for disease (Logan and Berg, 1996).

Host response to *H.pylori* and its clinical relevance

It is known that the resistance to infections can vary widely between individuals and may strongly influence the outcome of infection (Abel & Dessein, 1997). Interestingly, even in the populations with extremely high prevalence of *H.pylori* infection there are always individuals which are non-infected though it is likely that everyone has been exposed to the organism. This suggests that some individuals are either resistant (immune) to *H.pylori* or able to spontaneously eradicate the bacteria. This supports the cases when a small proportion of infected subjects loose their infection (seroconversion). The role of host factors in susceptibility to *H.pylori* infection was appropriately

demonstrated by the twin study (Malati et al., 1994). Among monozygotic twins 81% were infected compared to 66% for dizygotic twins (p=0.001). *H.pylori* infection and family history of peptic ulcer were shown the independent risk factors for peptic ulcer: OR 3.8 and 8.4, respectively (Brenner et al., 1998). However, if both factors were present the risk for peptic ulcer increased to OR-29.5 suggesting that several factors are involved.

Natural immunity

A principle mechanism of natural immunity to microbs is phagocytosis by neutrophils, monocytes, and tissue macrophages. Microbial LPS's were shown to activate the alternative complement pathway, in the absence of antibody, through the stimulation of macrophages and other cells to produce cytokines (TNFα, IL-1; IL-6, and chemokines) which stimulate inflammation (Bliss et al., 1998; Shimoyama and Crabtree, 1998). However, LPS of *H.pylori* was shown to have a much lower biological activity (Moran, 1996). Another effect of cytokines is a synthesis of HSP's and other acute phase proteins. It is to note that some of these cytokines (TNFα) may also stimulate T and B lymphocytes thus amplificating the specific immunity (Eigler et al., 1997). The NAP of *H.pylori* and urease are also potent stimuli of phagocyte activation and inflammatory cytokine production (Harris et al., 1996; Satin et al., 2000).

Naturally occuring antibodies that are able to bind self and nonself antigens, including those related to blood group epitopes, also participate in natural resistance against the infections (Blackwell, 1989; Bouvet & Digiero, 1998). In addition, there is a close relationship between natural immune mechnisms and acquired immunity: antibodies promote phagocytosis via opsonization of bacteria and activation of the complement thus enhancing an effector arm of the response (Haberle et al., 1995; Sommer et al., 1998). The effector immune mechanisms are nonspecific in terms of they can be induced by a great variety of inductors including infections.

Acquired immune response to *H.pylori*

In the majority of infected individuals, *H.pylori* elicits a strong systemic and mucosal immune response both humoral and cell-mediated. Given that infection persists for decades, it is clear that neither humoral nor cellular immune defences eliminate the organism. However, after *H.pylori* eradication re-infection in adults is uncommon (Mitchell et al., 1998; Gisbert et al., 1998), suggesting that immune mechnisms can prevent re-infection. In addition, protective immune response can be generated after vaccination with bacterial antigens together with appropriate adjuvants (Marchetti et al.,1995). From the

point of protective immunity, the membrane-associated proteins of *H.pylori* are more promising (Nilsson et al., 2000).

Systemic and mucosal immune response to *H.pylori* have been extensively studied in different clinical conditions but, despite it being useful for diagnostic purposes, the role of immune mechanisms in the pathothenesis and natural history of *H.pylori*-associated diseases remains to be clarified. Several main aspects of host immune response to *H.pylori* will be considered in the next sections: the type of the response, the antigens involved, the clinical relevance of the changes observed, their possible disease type specificity and relation to host factors.

Systemic and mucosal humoral immune response

The IgG antibody response to *H.pylori* is highly polymorphic in terms of its strength and antibody patterns. (Kist, 1991; Faulde et al., 1992, 1993; Crabtree et al., 1993; Fauchere, 1996; Nilsson et al., 1997, Enroth et al.,2000; Ng et al., 2001). Each patient appears to have his own pattern. As could be expected, the better diagnostic efficiency showed IgG immune response though only the IgA systemic response was found in some cases which were IgG negative. The crude and partially purified antigenic preparations were used as well as some recombinant fragments of *H.pylori* proteins such as CagA (Xiang et al., 1993; Kuipers et al., 1995a). The individual *H.pylori* strains showed fairly stable protein profiles after repeated subcultures (Morgan et al.,1991). In contrast, different strains exhibit wide qualitative and quantitative variations in antigenic profiles and in immunoblot reactivity especially in the molecular mass range from 50 to 70 kDa (Faulde et al.,1992, 1993; Enroth et al., 2000; Pineros et al.; 2001; Ng et al., 2001). These medium-size proteins (HSP60, flagellins etc.), were reported to be responsible for cross-reactivity with antigens of other bacterial species (Dunn et al.,1989; Andersen and Espersen, 1992; Engstrand et al., 1993; Nilsson et al., 1997).

With the exception of more defined and conserved *H.pylori* proteins (urease subunits, HSP's, flagellar antigens, CagA, VacA, and some bands with a lower molecular mass (25–30 kDa), the specificity of many protein bands for *H.pylori* is not fully proved. However, in spite of inter-individual variations in the immune response, the typical band patterns specific for *H.pylori* can be recognized on blots (Nilson et al., 1997; Aucher et al., 1998).

The CagA is highly immunogenic and the presense of serum anti-CagA antibodies strongly correlates with the cagA-positive status of the individual, and allows infection with the cagA-positive strains to be detected serologically (Cover et al., 1995). An enzyme-linked immunosorbent assay (ELISA) has been developed using recombinant fragments of CagA protein as antigen (Xiang et al., 1993; Blaser et al.,1995). Some ELISA negative patients with gastric cancer recognised the CagA band on blot (Crabtree et al., 1993). Such cases were more

frequent in areas with higher gastric cancer rates (Torres et al., 1998). Thus the prevalence of *H.pylori* infection is obviously underestimated in these patients. This implies that CagA antibody may detect past *H.pylori* exposure (Ekstrom et al., 2001; Enroth et al., 2001) whereas ELISA underestimates it.

Most authors found that IgM response to be neither specific nor sensitive in serologic diagnosis of *H.pylori* infection (von Wulffen et al.,1986; Faulde et al.,1993; Jones et al., 1986), especially in children (Blecker et al., 1995). It appears that IgM antibody is mostly directed to cross-reactive antigens shared by other Campylobacter species (Faulde et al., 1992; Andersen and Espersen, 1992).

Local IgA response is observed in all infected as determined in gastric mucosa extracts, gastric juice and in saliva. Bergquist et al., (2000) have shown a strong correlation between the anti-*H.pylori* IgA and IgG levels in the biopsy extracts and the frequencies of IgA-, IgG-secreting cells as detected with ELISPOT technique. At the same time, the systemic antibody response and saliva antibody levels did not correlate with the number of Ig-producing cells in the stomach. A similar pattern of IgA and IgG responses to *H.pylori* extracts or *H.pylori* urease was detected in serum, gastric juice and gastric mucosa tissue (Luzza et al., 1994; Futagami et al., 1998). Saliva, serum and gastric mucosa *H.pylori* antibody levels did not differ between superficial and atrophic, active and inactive *H.pylori* positive gastritis (Luzza et al., 1998).

None of the IgG antibody patterns as defined by immunoblotting were correlated to gastric atrophy (Aucher et al., 1998) though the simultaneous presence of 125,87 and 35 kDa bands predicted the risk of peptic ulcer disease with 83% sensitivity and 69% specificity. The IgG2 isotypic response was shown to be different in the patients with peptic ulcer or those with only gastritis (Bontkes et al., 1992). A recent study of Kimmel et al. (2000) revealed no association of specific *H.pylori* antigenic patterns with antibodies in patients with particular gastroduodenal pathology by two-dimensional gel electrophoresis. The ratio of H-pylori-specific IgA to total IgA in serum and gastric juice was correlated with the histologic grade of gastritis and no correlation was found for antibody titers to urease alpha and beta subunits (Hayashi et al.,1998). However, in a recent study (Pineros et al., 2001) where native strains were used, different clinical groups showed a tendency to react preferentially with antigens derived from the same disease. These authors also found that *H.pylori* antibody profile differed in prior and in active infections. Different *H.pylori* strains used as antigens in immunoblotting revealed appreciable variations in their ability to detect CagA in immunoblotting with the same set of sera, and reacted better when the *H.pylori* was derived from the patients with a similar pathology (cancer) (Vaucher et al., 2000). The asympthomatic individuals showed a lower CagA seroprevalence than patients with peptic ulcer and gastric cancer. These findings allow to assume that insufficient correlation between individual immune response to *H.pylori* and the disease type or natural course of the infection may be explained by inadequate

antigen used, i.e. standard strain of *H.pylori* instead of antigenic preparation from *H.pylori* isolate of a given individual.

Another important aspect is the strength of the immune response. The level of anti-*H.pylori* IgG antibodies as detected with ELISA varies enormously between infected individuals. Some subjects show 'gray zone' values whereas others reveal many times higher level of antibodies. However, these variations did not correlate strongly with the clinical symptoms or other manifestations of the infection. Several studies have demonstrated a correlation between the levels of total anti-*H.pylori* IgG and the colonization of the gastric mucosa by the bacteria (Pronovost et al., 1994; Kreuning et al., 1994; Hsu et al., 1997). The concentration of soluble *H.pylori* antigen and circulating specific *H.pylori* antigen immunocomplexes in serum was also shown to correlate with the number of *H.pylori* in gastric mucosa layer (Zhu et al., 2002). The density of *H.pylori* in both on the surface mucous cells and in the surface mucous gel layer correlated well with the severity of gastritis (Shimizu et al., 1996). *H.pylori* density of the antrum as well as grade of activity, inflammation and atrophy was significantly higher in duodenal ulcer patients than in chronic gastritis, gastric ulcer, and gastric cancer. (Kim and Back, 1999). In patients with gastric carcinoma, the density of *H.pylori* infection was decreased in proportion to advances in the cancer stage and the mucosal atrophy (Tokunaga et al., 2000), indicating that the prevalence of *H.pylori* may be affected by the stage of the disease. However, the levels of anti-*H.pylori* antibodies in patients with gastric cancer and age-matched controls showed no significant differences (Kuipers et al., 1993). But IgG antibodies for cagA gene product of *H.pylori* were more common in patients with both diffuse and intestinal type cancer than in uninfected controls: OR=10.1 and 5.1, respectively (Parsonnet et al., 1997).

The occurence of dyspeptic symptoms was also not related to the density of colonization (Braden et al., 1997). On the other hand, the duodenal ulcer risk was found to correlate with antral *H.pylori* density (Talamini et al., 1997). No difference in density between CagA(+) and CagA(-) *H.pylori* strains neither no difference in topographic localization of these strains was noted (Twisk et al., 2001). But in another study, a higher IgG antibody titer was found in patients harboring cagA-positive *H.pylori* strains (Loffeld et al., 2000b).

Interestingly, the prophylactic immunization against *H.pylori* is equally effective in mice deficient in IgA and B lymphocytes whereas a positive effect of therapeutic immunization of B-cell deficient infected mice was antibody independent (Sutton et al., 2000; Ernst and Pappo, 2001). It is notable in this respect that Th1 type immune response is induced early during acute *H.pylori* infection in Rhesus macaques (Mattapallil et al., 2000).

Thus, many controversies still exist regarding the role of immune response to *H.pylori* in host-microbe interplay and it remains unclear, whether systemic or local humoral immune response might adequately reflect natural course of the infection or its clinical outcome.

Cell mediated immune response

A number of studies have shown that natural *H.pylori* infection leads to a Th1-type response (Mohammadi M et al. 1996; Bamford et al., 1998; Ren et al., 2000) which is contributed to delayed-type hypersensitivity and may lead to the mucosal damage *via* stimulation of the innate immune system, e.g. non-antigen specific NK cells and mucosal macrophages, leading to production of proinflammatory cytokines such as IL-1β, IL-6, IL-8, TNFα and IFN γ (Crabtree et al., 1993, 1994a; Haberle et al., 1995; D'elios et al., 1997; Glupczynski & Devaster, 1997; Sommer et al. 1998; Kuipers, 1999; Nakachi et al., 2000;). In contrast, a lower level of anti-inflammatory (Th2) cytokines was demonstrated (Fan et al., 1995). However, the changes in the T-cell response of blood leukocytes did not always reflect those in the mucosa and may be increased irrespective of *H.pylori* status (Birkholz et al., 1993, Fan et al., 1995; Karttunen et al., 1997). It has been shown that gastric mucosa T cells are polarized to produce Th1 cytokines in the absence of *H.pylori* (Itoh et al., 1999). Bodger et al. (1992) reported that, in addition to an increased production of TNFα, there was also higher secretion of anti-inflammatory and immunosuppressive IL-10 in *H.pylori* infected mucosa that may be protective for *H.pylori*. Ren et al., 2001 observed a shift from a Th1-type to a Th2-type response (increased IL-4 production and decreased IFN gamma production) in patients with gastric cancer and dysplasia.

There are close interactions between the immune system and gastric epithelial cells in *H.pylori* gastritis. This includes the the upregulation of interleukin-8 and de novo expression of MHC class-II antigen in gastric epithelium. (Engstrand et al., 1989, Crabtree et al., 1993, 1994b). In gastric epithelial cell cultures *H.pylori* infection results in increased IL-8 levels which correlate with neutrophil infiltration (Crabtree et al., 1993). The ability of CagA(+) strains of *H.pylori* to induce IL-8 transcription correlated with the ability of the strain to activate nuclear factor NF-kB indicating a mechanism for a higher inflammatory response seen in individuals infected with CagA(+) *H.pylori* strains (Sharma et al., 1998). Release of lysosomal enzymes and oxygen free radicals by neutrophils during phagocytosis of bacteria may further contribute to mucosal damage and carcinogenesis (Dunn et al., 1993). Moreover, IFNγ and TNFα produced by gastric Th1 cells can induce epithelial cell death or increase apoptosis induced by *H.pylori* (Wagner et al., 1997; Fan et al., 1998). LPS of *H.pylori* induces apoptosis of gastric epithelial cells in rats (Piotrowski et al., 1997) and in human epithelial cells in vivo (Jones et al., 1996). The strains expressing the cagPAI induce greater level of inflammation (Yamaoka et al., 1997) suggesting that the variation in the host response may explain a more severe disease in individuals infected with CagA+ strains. However, a recent study of Rad et al. (2002) showed that mRNA amounts of the Th1 markers did not differ between patient groups infected with different strain types.

All these data brought to a speculation that the elimination of *H.pylori* is dependent on a Th2 response which helps to promote antibody responses. It seems, however, that this is oversimplification because mice deficient for IFN-γ (where Th2 response is dominated) have showed no protective response after immunization (Sawai et al., 1999). Another study showed that *H.felis* infection was even slightly increased in IL-4 (Th2 cytokine) knockout mice (Chen et al., 1999).

However, recent investigations in mice deficient in IgA or B cells showed that they were capable to elicit effective immunity and provide protection (Blanchard et al. 1999; Ermak et al., 1998). This points to the fact that other effector mechanisms (except B cells or antibody) are also important in providing immunity and protection to luminal pathogen (Ernst et al., 2001). Interestingly, after immunization, the antigen-specific response is predominantly polarized toward a Th2-type response, with production of cytokines that can inhibit the activation of Th1 cells and of macrophages, and the production of proinflammatory cytokines (Del Guidice et al, 2001).

A general conclusion which can be made from all these studies is that the ability of an individual to respond to *H.pylori* with specific immune mechanisms can appreciably affect the severity of disease and the risk of progressing disorders such as atrophy and cancer. But it seems that none of these mechanisms are strongly disease-specific. However, the peculiarities in the host response to *H.pylori* appear to be important determinant. The IL-1 gene polymorphism in the interleukin-1 gene cluster is a very attractive example. The gastric cancer relatives with hypochlorhydria had a significantly higher frequency of the proinflammatory IL-1RN*2 allele and T-T haplotype of IL-1β-31 and IL-1β-511, as compared to those without hypochlorhydria (El-Omar et al., 2000; El-Omar, 2001) suggesting that host pro-inflammatory IL-1 genotypes are important in determining the functional response to *H.pylori* infection, the pattern of gastritis and the ultimate clinical outcome of gastric cancer.

H.pylori infection and autoimmunity

Immunity, like many other homeostatic mechanisms, is beneficial for the host but also has a potential to be detrimental to him. Injury of adjacent normal tissue is a pathologic side effect of these defence mechnisms.

Evidence is accumulating that an ongoing immune stimulation by *H.pylori* antigens may lead to the autoimmune reactions of various specificity. Antibodies to several target antigens may be involved: autoantibodies to the surface of foveolar epithelial cells, mucosal neck cells, anti-canalicular antibodies to the canaliculi of parietal cells, intrinsic factor, to the Lewis antigens and HSP's (Negrini et al.,1991; Maccia et al., 1993; Uibo et al., 1995; Appelmelk et al.,1996; Faller et al., 2000; Vorobjova et al., 2000). These

reactions were shown to correlate with the rate of apoptosis in the gastric mucosa (Kirchner et al., 1997). This mechanism may represent a pathogenic link between *H.pylori*-related gastritis and atrophy. In an immunohistochemical study, many monoclonal antibodies against *H.pylori* were reacted with gastric epithelial cells (Ko et al., 1997). The cross-reacting antigens included urease, flagella, LPS and heat shock protein of *H.pylori*. H+K+ATPase is a major target antigen in autoimmune gastritis (D'Elios et al., 2001). The beta subunit of *H.pylori* urease was shown to have a high homology with gastric H+K+ATPase, the gastric parietal cell antigen (Uibo et al., 1995). These authors also found a negative association between *H.pylori* and autoimmune corpus gastritis. In another cohort study, a significant increase in parietal cell antibody positivity was observed among *H.pylori*-infected individuals (Vorobjova et al., 2000). The presense of common or cross-reacting epitopes for gastric mucosa cells and *H.pylori* is considered as an important pathway for induction of autoimmunity through the molecular mimicry mechanism (Negrini et al., 1991,1997). Molecular mimicry of autoantigens by microbes can stimulate autoreactive cells by their cross-reactivity in individuals where there are defects in immunoregulation. A strong expression of class II transplantation antigens and an increased number of γ/δ T-cells was demonstrated in gastric epithelial cells of *H.pylori*-infected individuals suggesting an increased possibility for initiation of local immune response and autoimmunity (Engstrand et al., 1989, 1991). However, in spite of a significant correlation found between anti-gastric autoantibodies and gastric mucosa atrophy (Valle et al.,1996; Claeys et al., 1998;Vorobjova et al., 2000) it remains unclear whether these are the cause or the sequel of gastric atrophy.

An inflammation induced by *H.pylori* leads to an enhanced expression of HSP in human gastric epithelial cells (Engstrand et al.,1991). The presence of anti-HSP antibodies was significantly correlated with the grade of chronic inflammation, associated with the progression of corpus gastritis (Valle et al., 1996) but was not related to the development of atrophy during 18 years of follow-up (Vorobjova et al., 2001). In an autologic system, Hsp60 was shown not to be likely a target for autoimmunity in *H.pylori* infected individuals whereas Le(x,y) epitopes did (Taylor et al., 1999).

Possible involvement of blood group related antigens in autoimmunity is reviewed in the next section.

H.pylori and gastric glycoconjugates

The gastric mucus and mucosal cell surface glycoconjugates constitute normal microenvironment for *H.pylori*. Mucins are highly glycosylated in O-linkage to serine and threonine residues (O-glycans), high molecular weight glycoproteins produced by many epithelial cells and tumors (Lesuffleur et al., 1994; Gendler et al.,1989). Most of them are secretory or gel-forming mucins except MUC1

which is a membrane-bound mucin (Van Klinken et al., 1995). Normal gastric mucosa expresses MUC1 and MUC5AC mucins in foveolar epithelium and MUC6 in the glands (Pinto-De-Sousa et al., 2002).

Expression pattern can be modified under pathological conditions (Silva et al., 2002). Besides, mucins show great heterogeneity at the carbohydrate level (Lesuffleur et al., 1994) that correlates with Lewis antigen expression in the human stomach (De Bolos et al., 1995). Tumor-associated mucins are different, in that they are aberrantly (incompletely) glycosylated, leading to the exposure of the protein core and to the expression of more short and less-branched carbohydrate side chains that constitute several carbohydrate tumor-associated epitopes such as blood-group-related antigens Tn (GalNAcα-O-Ser/Thr), sialyl-Tn and Thomsen-Friedenreich (T) antigen (Galβ1-3GalNAcα/β-O-Ser/Thr) (Hakomori, 1989; von Mensdorff-Pouilly et al., 2000a). Natural antibodies to these, as well as to peptide epitopes exposed due to altered glycosylation of the mucin molecule, are present in every individual. The level of anti-carbohydrate antibodies is rather stable for a given individual but varies appreciably between individuals for yet unknown reasons. Natural immune response to these epitopes is considered to be one of the natural defence mechanisms against cancer (Springer, 1984; von Mensdorff-Pouilly et al., 2000b).

Blood group ABH and Lewis (Le) antigens are expressed in gastric glycoconjugates in both cell membrane-bound and secretion. The expression of blood group-related carbohydrate epitopes on gastric mucins and on epithelial cell glycoconjugates has been shown to be dependent on secretor/nonsecretor (Se/se) status (Sakamoto et al., 1989; Oriol et al., 1992). The secretor status is determined by the presence or absence of a fucose residue on the terminal galactose residues on the mucosal O-linked oligosaccharides and mucins in secretions, which in turn is determined by the inheritance of the appropriate α-1-2 fucosyl-transferase *Se* (secretor) gene (Oriol et al., 1992; Henry and Oriol, 1995). At the phenotypical level the secretors belong to Le (a-b+) and those who are non-secretors to Le (a+b-) histo-blood-group phenotype. Individuals with Le (a-b-) phenotype may be secretors or non-secretors depending on the *Se* gene which are present in about 90% of them (Henry and Oriol., 1995). An expression of some other carbohydrate antigens is also related to *Se/se* status. In particular, the difference concerns the expression of cancer related T epitope on type 3 mucin-type chains in non-secretors exclusively (Bara et al., 1993). In contrast, in secretors this epitope is further glycosylated (Okada et al., 1994). Normal surface gastric epithelia express T in alpha-anomeric configuration (Sotozono et al., 1994). An expression of T epitope was reported to be increased in the cytoplasm of surface and glandular mucous cells in *H.pylori* infected patients with chronic gastritis (Barresi et al., 2001).

Gastric mucosa cells also express Le type 2 epitopes Le(x) and Le(y) but histochemically the topography is different. No Le(x) expression was found in the foveolar epithelial cells of either secretors or nonsecretors whereas some amount of Le(y) can be detected in this area in secretors. However, both

determinants were demonstrated in gastric glands irrespective of Se/se status (Sakamoto et al., 1989). Amano et al. (1997) reported on the presence of natural antibody to Le type 2 antigens in the blood of every individual irrespective of *H.pylori* status. It is not known whether the immune response to Le(x) and Le(y) is related to the host Lewis phenotype. Anti-Lewis(x) IgG was detected more frequently in dyspeptic subjects compared to healthy individuals, whereas the prevalence of IgM Le(x) antibodies was higher among healthy people and might have protective role (Rudnicka et al., 2001). *H.pylori*-infected individuals with a low level of anti-Le(x) antibodies were shown to be at a higher risk for gastric mucosa atrophy development (Kuipers et al., 1997). No relation of anti-Le(x,y) antibodies to anticanalicular autoantibodies was demonstrated in absorbtion experiments indicating that other mechanism than molecular mimicry leads to the formation of parietal cell antibodies (Faller et al., 1998).

Many blood group antigen related carbohydrate epitopes have been shown to be expressed in bacteria (Blackwell, 1989). On the other hand, some of carbohydrate antigens of gastrointestinal mucosa may be a target for microbial adhesins (Rios and Bianco, 2000). The Le (b) blood-group antigen is known to mediate the *H.pylori* attachment to human gastric mucosa (Boren et al., 1993) *via* blood group antigen binding adhesin (BabA) of *H.pylori* (Gerhard et al., 1999; Printz et al., 2001) suggesting that the Le(a+b-)/non-secretors might be more resistant to *H.pylori* infection than secretors. However, many researchers found no association between the prevalence of *H.pylori* infection and the proportion of non-secretors or the distribution of Lewis blood group phenotypes in dyspeptic individuals and patients with peptic ulcers (Höök-Nikanne et al., 1990; Mentis et al., 1991; Chesner et al., 1992). Interestingly, blocking of *H.pylori* Le(b) with anti-Le(b) monoclonal antibodies did not alter the binding of the bacteria to Le(b) thus suggesting that expression of Le(b) in *H.pylori* does not interfere with the bacterial adhesion property to immobilized Le(b) (Zheng et al., 2003). BabA is supposed to facilitate colonization of *H.pylori* and increases IL-8 response resulting in enhanced mucosal inflammation (Rad et al., 2002). The *vacAsI+/cagA+* strains harboring *babA2I* were associated with a higher degree of inflammation and colonisation than *vacAsI+/cagA+* strains lacking *babA2*. In addition to Le (b) antigen, the H-1 disaccharide (Fucα1-2Gal) was shown to be the minimal receptor for *H.pylori* adhesion (Boren et al., 1994).

The majority of *H.pylori* strains was shown to express human blood group antigens ABH, Lewis type 1 and type 2 antigens (Sherburne and Taylor, 1995; Appelmelk et al., 1996; Wirth et al., 1997). *H.pylori* can adapt its pattern of Lewis antigen expression in LPS to mimic that of its host, indicating that the pathogen is able to regulate this virulence factor (Wirth et al., 1997). However, other researchers (Taylor et al., 1998; Henegan et al., 2000) did not find a concordance between bacterial and host expression of Le determinants though the last authors showed that Le(x) expression was associated with a higher density of colonization and inflammation. It may be related to the phenotypic

diversity of Le antigen expression in *H.pylori* isolates derived from the same host (Wirth et al., 1999). The Lewis antigen expression in *H.pylori* is also related to CagA status (Wirth et al., 1996). No apparent association between Lewis phenotype of *H.pylori* isolate and disease pathology was evident in another recent study (Ryan et al., 2000). On the other hand, Zheng et al., (2000) demonstrated that, in Asian population, peptic ulcer was associated with the increased expression of Le antigens, but not with CagA status, or iceA or vagA genotypes in *H.pylori* isolates.

It has been shown that IL1β and IL-6 stimulated mucus secretion, while IFN-γ potentiates *H.pylori*-decreased mucus secretion by gastric epithelial cells (Takahashi et al., 1998). *H.pylori* was shown to inhibit total mucin synthesis *in vitro* and decrease the expression of MUC5AC and MUC1 mucins (Byrd et al., 2000). There is an evidence that VacA is involved in down-regulation of mucin expression or secretion (Beil et al., 2000). An aberrant expression of Le(x) in surface mucous cells has been demonstrated in *H.pylori* infected subjects (Byrd et al., 1997). The prevalence of Le(y) expression was found in patients with duodenal ulcer (Thoreson et al., 2000). The glycosylation of gastric mucins has been shown to be reversibly altered by *H.pylori* (Ota et al., 1998). These findings may be related to the glycosidase and mucinase activity of *H.pylori* (Smith et al., 1994; Dwarakanath et al., 1995). *H.pylori* possesses a gene that is almost identical to a mucinase gene of Vibrio cholerae (Smith et al., 1994). Possible mucinase activity as well as a degradation of the gastric mucus by the phospholipase A (Xerry and Owen, 2001) may be contributed to the damage of gastric mucosal barrier.

An over-representation of blood group A individuals among patients with gastric cancer and the prevalence of blood group O subjects and those who are non-secretors among patients with peptic ulcers were shown long ago (Aird et al., 1954; Correa et al., 1973; Mourant et al.,1978; Mentis et al., 1991), but the basis for this association remains unknown. A higher proportion of blood group O subjects but no relation to Lewis type or Se/se status was found among *H.pylori*-infected patients with dyspeptic symptoms who underwent endoscopy (Mattos et al., 2002). It has been reported that the leukocytes of blood group O donors revealed a stronger inflammatory response to *H.pylori* and released significantly higher amount of IL-6, TNFα and nitric oxide than blood group A leukocytes (Alkout et al., 2000).

Altogether, it appears that heterogeneity of *H.pylori* and gastric glycoconjgates is contributed to the pathogenesis of *H.pylori* infection and related to immunologic response of the host to blood group related carbohydrate epitopes. It is not clear to what extent these mechanisms are contributed to natural course of the infection or its clinical outcome.

BACKGROUND AND AIMS

An enormous genetic and phenotypic polymorphism of both *H.pylori* and host suggests the heterogeneity of outcomes between the patients. It is still not clear yet to what extent and how bacterial virulence and to what extent the genetic disposition of the host contribute to this issue. A growing body of evidence suggests that a majority of *H.pylori* pathogenetic pathways are strongly related to immunologic mechanisms especially to the effector arm of host immune response. Humoral immune response to *H.pylori* may be considered as an integral indicator of *H.pylori*-host interplay in the sense that it reflects both the *H.pylori* antigenic (phenotypic) polymorphism and the host's ability to respond to *H.pylori* infection which is a typical 'slow infection' where the host is the major determinant. Therefore, on the basis of the evidence to date, further studies are required to resolve the role of host's polymorphism in immune response to *H.pylori* and pathogenesis of *H.pylori*-associated diseases.

An insufficent correlation between the immunological response to *H.pylori* and clinical outcome of the infection may be addressed to several questions: (i) to what extent the differences in the immune response are dependent on host polymorphism and to what extent on *H.pylori* diversity? (ii) Is it possible to select the disease-type peculiarities of the immune response and to predict the clinical outcome of the infection? And (iii) can this decision be based on immunological criteria on the individual level?

In the present investigation special attention was paid to the phenotypic traits of the host that may influence *H.pylori*-host immunological interaction. These include histo-blood group related antigens and their derivatives which were reported to be expressed on both *H.pylori* and host gastric mucins and mucosal cell glycoconjugates. Mucins represent normal in vivo microenvironment for *H.pylori* and undergo appreciable alterations in gastric disease. In the host, this is a highly polymorphic system that is controlled by *ABH, Le, H* and *Se/se* genes and involved in *H.pylori* adhesion and colonisation. Besides, these antigens may be the target for induction of autoimmunity which is closely related to gastric mucosa damage and thus to the clinical outcome of the infection. A natural and acquired immune response to these carbohydrate epitopes may reflect both their expression in *H.pylori* and the ability of the host to recognize them, and to respond to them. And finally, we explored a hypothesis that *H.pylori* might be indirectly involved in gastric carcinogenesis *via* systemic impact on naturally occuring immune mechanisms against cancer.

Aims

In the present study, the broad objective was to investigate the humoral immune response to *Helicobacter pylori* antigens in patients with gastroduodenal

diseases and in blood transfusion donors with special attention to the host phenotypic polymorphism for blood group related antigens which may contribute to the pathogenesis and clinical outcome of *H.pylori*-associated diseases.

The specific aims of the thesis were:

- To evaluate the seroprevalence of *H.pylori* infection and more virulent CagA positive *H.pylori* strains in the patients with gastroduodenal pathology (gastric cancer, peptic ulcer disease and chronic gastritis) and blood transfusion donors in relation to ABH, Lewis blood group phenotype or *Se/se* secretory status of the host. To assess whether these phenotypic characteristics of the host may cause the differences in the seroprevalence of *H.pylori* infection and contribute to the disease type-specific features of the immune response to *H.pylori* antigens.
- To investigate the effect of *H.pylori* infection and host Le(a,b) phenotype on natural immune response to Lewis type2 antigens.
- To study possible systemic impact of *H.pylori* infection on natural immune response to tumor-associated blood group-related Thomsen-Friedenreich antigen (Galβ1,3GalNAcα/β-O-Ser/Thr) in different clinical groups.
- To test the hypothesis of whether *H.pylori* might express the tumor-associated Thomsen-Friedenreich antigen.
- To evaluate the survival of patients with gastric cancer depending on *H.pylori* serologic status and on the level of natural antibody to tumor-associated Thomsen-Friedenreich antigen.

MATERIAL AND METHODS

The detailed description of the material and methods used are described in the corresponding papers (I–VIII). However, some additional methodological aspects are presented in this section to stress some methodological points in more detail that were not fully described due to the limited space of journals.

The results were analysed in two ways: by comparison (i) between the *H.pylori*-seronegative and -seropositive subgroups within each group (donors, patients) to look for a relation of the changes found to *H.pylori* infection, and (ii) between the groups for both infected and non-infected individuals trying to find putative disease type-specific features. Host-dependent factors such as ABO(H), Lewis(a,b) phenotype, secretory (Se/se) status, a level of T antigen-specific IgG, IgM antibodies, anti-Le type II antibodies, the disease type, tumor morphology and the stage of cancer, the survival of patients with cancer were considered as predictors and correlated with *H.pylori* serologic status, the level of anti-*H.pylori* antibodies, the CagA status, the immunoblot pattern, and the expression of tumor-related T antigen in *H.pylori*.

Subjects

The patients with benign gastric diseases (n=204) and those with histologically verified gastric carcinoma (n=281) were two main study groups. In addition, a group of randomly selected blood transfusion donors (n=306) were studied as the control group to determine the ABH and Lewis phenotype distribution among *H.pylori*-positive and negative subgroups of individuals, and to evaluate the impact of these blood group antigens polymorphism on the other parameters. Individuals of younger age (below 40 yrs) were not included in some parts of the study (IV, V) to make the distribution by age comparable to that of patients with gastric cancer.

Tumor staging and morphology were based on histopathological (pTNM) classification of malignant tumors (Sobin and Wittekind, 1997) and evaluated according to the system of Lauren (1965) as intestinal and diffuse type of tumor growth. Peptic ulcer disease was diagnosed by gastroduodenal endoscopy. In patients with chronic gastritis, antrum and corpus biopsy specimens (aa 1–2 specimens) were assessed histologically in accordance with the updated Sydney system (Dixon et al., 1996). Moderate or severe gastric mucosa atrophy in any part of the stomach was considered as atrophic gastritis. The distribution of cancer patients by stage and other characteristics of individuals studied are presented in papers I–VIII.

H.pylori strains and culture conditions

H.pylori strain NCTC 11637 (CagA and VacA positive) was cultured on Gonococcal agar base/Campylobacter (GAB/CAMP) reference agar (Oxoid Ltd, Basingstoke, Hampshire, England) supplemented with 10% horse blood, selective supplements (Dent) and IsoVitaleX Enrichment (Oxoid) at 37°C for 2–3 days under microaerophilic conditions (5% O2, 10% CO2, 85% N2). The clinical isolates of *H.pylori* from antral gastric mucosa were cultured in a similar way. The strains were stored at –70°C in Tryptic Soy Broth containing 15% (v/v) glycerol.

Extraction of H.pylori cell surface proteins

A glycine cell surface membrane antigens extraction (0.2M acidic glycine, pH 2.2, for 15 min.) of *H.pylori* strain NCTC 11637 and *H.pylori* clinical isolates was performed according to Logan and Trust, 1983. Extracts were stored at –70°C and used as an antigen for ELISA and SDS-PAGE within 6 months. The antigen was a pool of 5–9 different preparations. The individual clinical isolates of *H.pylori* were treated in a similar way.

H.pylori enzyme-linked immunosorbent assay (ELISA)

Flat bottom micrititer plates (Maxi Sorp, Nunc, Roskilde, Denmark) were coated with 0.1 ml of antigen (5μg/ml in carbonate buffer pH 9.6) per well and incubated at 4°C overnight. Alkaline phosphatase conjugated goat anti-human IgG (Gibco BRL, USA or Dako A/S, Glostrup, Denmark) and p-nitro-phenyl-phosphate (Sigma, St.Louis, MO) as substrate were used. A pool of human IgG (Kabi AB, Stockholm, Sweden) was placed in each ELISA plate as a positive control for 100 units and *H.pylori* seronegative reference serum was run for negative control. The relative antibody activity (RAA) values were calculated according to Blomberg et al., 1983. RAA values ≤25 were regarded as *H.pylori* seronegativity. In control experiments, the method was compared with H.pylori ELISA commercial kit (Pyloriset EIA-G, Orion Diagnostica, Finland) in 36 blood donors (10 seronegative): the coincidence of results was observed in 34 of 36 (94.4%) subjects. In some studies, (V,VI) to improve the discrimination between *H.pylori* infected and noninfected individuals, the subjects with a 'gray zone' RAA values (RAA in the ranges of 26–39) were excluded from the analysis.

Blood group phenotyping and secretor/non-secretor (Se/se) status evaluation

ABO(H) phenotyping of erythrocytes was carried out using anti-A and anti-B monoclonal antibody (MonoCarb AB, Sweden). Lewis phenotyping of erythrocytes was carried out using anti-Le(a) and -Le(b) monoclonal antibody gel system (DiaMed, Switzerland) according to the instruction of the manufacturer.

In the patients with benign gastric diseases the secretor/non-secretor (*Se/se)* status was determined by tesing of H-antigen in boiled saliva at dilution 1:10 using H-specific biotin-labelled lectin UEA-1 from *Ulex europaeus* (Sigma) as described by Rahat *et al.*, 1990. The method was compared with Lewis phenotype determined by erythrocyte testing in 28 individuals and the results were identical in 96% of cases among the subjects of Le(a+b-) /non-secretor and Le(a-b+)/secretor phenotype. All non-secretors showed O.D. values below 0.25 (mean ± 2t·S.E. at P=0.05). This O.D. value was used as a cut-off limit to discriminate between the secretors and non-secretors. No overlapping for O.D. values was observed in both groups except one donor of Le(a-b+) phenotype. He was classified as Le(a-b+)/secretor as detected with MAb but defined as non-secretor by UEAI lectin assay. Apparently he belonged to the so-called 'weak' secretors (Oriol et al., 1992).

H.pylori cell-ELISA

Freshly prepared from 2 days culture *H.pylori* cells were fixed with 0.5% glutaraldehyde for 20 min at RT. After being blocked with 0.15 M glycine and 1% bovine albumine and washing 3 times with phosphate buffered saline (PBS) pH 7.4, 10^8 cells per tube in 0.1ml of PBS were incubated with equal volume of T antigen-specific MAb dilutions for 2 hrs at room temperature (RT). Bacteria were then washed four times in PBS-0.05% Tween-20 and incubated with either alkaline phosphatase-conjugated rabbit anti-human IgM or, for murine MAb, with biotinylated goat anti-mouse immunoglobulins (Dako, Denmark) for 1 hr at RT followed by streptavidin-alkaline phosphatase conjugate (Dako) for 1 hr. After additional 3 washes, the bacteria were incubated with p-nitro-phenyl-phosphate solution (Sigma, St.Louis, MO) for 30 min. Absorption was read at 405 nm. Bacteria incubated with PBS instead of MAb were treated in the same way and served as control. The assay was performed in duplicate.

Sodium-Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

SDS-PAGE was performed in 12% polyacrylamide separating gel according to Laemmli, 1970. Nitrocellulose sheets (Schleicher&Schuel, Germany) or PDF

membrane Immobilon P (Millipore, Bedford, MA) were used for immunoblotting. After separation and semidry transfer in Hoefer vertical electrophoresis equipment (Amersham, Pharmacia Biotech, Finland) the membranes were saturated with blocking buffer recommended by Rucheton et al. (1992) (glycine, hydrolysed gelatine, Tween 20, polyvinyl-pirrolidone, ethanolamine), that results in very low background even at long staining time. After incubation with diluted serum or monoclonal antibodies or antiserum the strips were washed and incubated with either alkaline posphatase or peroxidase-labelled anti-human or anti-mouse IgG, IgM (Gibco, Dako) and, after repeated washing, developed with NBT/BCIP substrate (Sigma) or with 3-amino-9-ethylcarbasol, respectively (Sigma). All *H.pylori* extracts were tested under the same electrophoretic and blotting conditions, serum and MAb's dilutions etc. For semiquantitative evaluation, the strips were scanned (Sharp image scanner JX-330) and the relative proportion of bands was calculated using Image Master TotalLab software (Amersham Pharmacia Biotech, Finland).

Evaluation of CagA serologic status

The CagA serologic status was assessed by two methods:

1. After electrophoretic separation of cell surface membrane proteins of *H.pylori* and blotting the membranes were cut into strips, washed with PBS and incubated with patients serum (1:76) in PBS-Tween 20 (0.05%) overnight at 4°C under agitation. After washing the strips were incubated with peroxidase labelled rabbit anti-human IgG (Gibco) and developed with 3-amino-9-ethylcarbasol (Sigma). A positive reaction was defined as the presence of a specific CagA band of ~120 kDa on the blots. The CagA-seronegative and CagA-seropositive serum were simultaneously run as controls. In addition, the distribution of other major protein bands of *H.pylori* on blots was evaluated in patients with gastric cancer and blood donors.
2. CagA–ELISA was performed as described by Xiang et al., 1993 with slight modifications (IV). Flat-bottomed polystyrene plates (Maxi Sorp, Nunc) were coated with 100 µl/well (1.25 µg/ml) of the purified recombinant fragment coding for the immunodominant region of the CagA protein, expressed as a fusion protein in *Escherichia coli* and purified as described by Xiang et al., 1993. An alkaline-phosphatase-conjugated goat anti-human IgG (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) and p-nitrophenyl phospate (Sigma) as substrate were used. The cut-off limit equal to an absorbance of 0.42 was determined on the basis of the investigation of 26 *H.pylori*-ELISA and CagA blot-negative and of 46 CagA blot-positive blood donors sera. This cut off value gave the best discrimination between the groups and the O.D. values more than O.42 were regarded as positive reaction (strong responders). Two reference sera from CagA-seropositive

and CagA-negative donors, respectively, were included in each plate as internal standards to control the interassay variations. A sensitivity of 96.2% and specificity of 96.6% has been reported for this assay as compared with Western blot (Xiang et al., 1993).

Determination of serum anti-Thomsen-Friedenreich (T) antigen specific antibodies by ELISA

Plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with synthetic T-hapten-polyacrylamide (PAA) conjugate (Synthesom, Munich, Germany; 10 mol% of carbohydrates) 5µg/ml in carbonate buffer, pH 9.6 or 1% BSA in PBS (control wells) at 4°C overnight. After washing three times with PBS-Tween the plates were blocked with 0.15ml of 1% BSA in PBS for 1hr at room temperature (RT) and washed in PBS-Tween. Serum (100µl) diluted 1:50 in PBS-Tween were added and incubated for 2 hrs at RT. The plates were then washed and bound IgG was detected with 100µl of alkaline phosphatase conjugated goat anti-human IgG or IgM (Gibco, BRL, Life Technologies, Gaithersburg, MD, USA) diluted 1:1000 in PBS-Tween. Following the incubation of 90 min at RT and washing, the plates were developed with *p*-nitro-phenyl-phosphate (Sigma) and absorbance values at 405 nm were registered with Labsystem Multiscan MCC/340 (Finland). The optical density (O.D.) values of control wells (PBS-BSA) were subtracted from the values of the wells coated with the T conjugate. Each serum was analysed in duplicate. The intraassay variations did not exceed 7%. For more details, see paper VII.

Depending on the O.D. values individuals were divided into 'strong' and 'weak' responders. The cut-off limits for IgG and IgM antibody levels were calculated on the basis of the O.D. values distribution and allowed the best discrimination between subjects with low and high O.D. values. The distribution curves were optimized using CurveExpert v.1.2. program as described elsewhere (Kurtenkov et al., 1999).

Expression of Thomsen-Friedenreich (T) antigen in H.pylori

Cell surface membrane extracts of *H.pylori* standard NCTC 11637 strain and the clinical isolates of *H.pylori* (n=13) were analysed by immunoblotting and cell-ELISA with five different T antigen-specific murine and human monoclonal antibodies (VII). The specificity of immunostaining was checked in blocking experiments using peanut agglutinin and rabbit antiserum to T antigen. Anti-H type 2 and anti-CD-43 Mab's were used as isotypic controls.

In *H.pylori* cell-ELISA, freshly prepared from 2 days culture *H.pylori* cells were fixed with 0.5% glutaraldehyde for 20 min at RT. After being blocked

with 0.15 M glycine and 1% bovine albumine and washing 3 times with PBS, 10^8 cells per tube in 0.1ml of PBS were incubated with equal volume of T antigen-specific MAb dilutions for 2 hrs at RT. Bacteria were then washed four times in PBS-0.05% Tween-20 and incubated with either alkaline phosphatase-conjugated rabbit anti-human IgM or, for murine MAb, with biotinylated goat anti-mouse immunoglobulins (Dako, Denmark) for 1 hr at RT followed by streptavidin-alkaline phosphatase conjugate (Dako) for 1 hr. After additional 3 washes, the bacteria were incubated with p-nitro-phenylphosphate (Sigma, St.Louis, MO) for 30 min. and the absorbance was read at 405 nm. Bacteria incubated with PBS instead of MAb were treated in the same way and served as control. The assay was performed in duplicate.

H.pylori eradication therapy

The patients with duodenal ulcer were treated with a standard one week triple therapy (amoxycillin 1.0g, clarithromycin 0.5g, and omeprasol 20mg twice a day). All the treated patients and untreated control subjects were followed for 6–24 months. Serum samples taken before and at different intervals after therapy were stored at –20°C and tested in parallel. The decrease of IgG *H.pylori* antibody level (RAA) at 6 month after therapy more than by 50% was considered as a sign of successful treatment. This group was used for evaluation of *H.pylori* eradication impact on the level of T antigen specific antibodies.

Statistical methods

Statistical comparisons between the groups were performed by the Student's t-test and Pearson two-tailed correlation. Nonparametric chi-square (χ^2) test and Fisher's exact test (for small groups) were applied for comparison groups discriminated by cut-off limits. Odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated to measure the association between the studied parameters. The difference was considered to be significant when $P<0.05$.

The cumulative survival of cancer patients was estimated by the Kaplan-Meier method, and the resulting curves were compared by the use of the log-rank test (SPSS software, version 10.0.5). Mean and median survival time with the standard error and 95% confidence interval was calculated for every group of patients divided by *H.pylori* status and/or anti-T antigen specific antibody level. Statistical tests were two-sided and the difference in survival between the groups was considered significant if $P<0.05$.

RESULTS AND DISCUSSION

H.pylori seroprevalence

IgG immune response to acid glycine extracted cell surface proteins of the *H.pylori* strain NCTC 11637 was studied in different groups of patients and blood donors to characterize the background seroprevalence parameters for further analysis of their relation to ABH and Le phenotype of the host. The individuals over 40 years of age were over represented to make the groups comparable to patients with gastric cancer.

The *H.pylori* seroprevalence was high and strickingly similar in blood transfusion donors and patients with gastroduodenal pathology, being in the ranges of 74–86%. In the blood donor group, it was 79.1–83.6% in three consecutive studies (paper I–III). These data are in concordance with the findings observed in southern Estonia with no significant age-related differences from 20–90 years (Vorobjova et al., 1994; 1998). Such similarity suggests that the results reflect the real rate of *H.pylori* seropositivity in the population.

No appreciable disease type-specific differences in *H.pylori* seroprevalence were found between the groups studied. The only exception was a significantly lower seroprevalence in patients at advanced stages of gastric cancer compared to the seropositivity rate in blood donors, patients with I+II stage cancer (paper I,V) and patients with duodenal ulcer (V). No relation to the morphological type of tumors (diffuse or intestinal type according to Lauren classification) was observed (I). These findings may be explained by the fact that very high seroprevalence is characteristic of the Estonian population. Higher *H.pylori* prevalence in patients with more severe gastric pathology (peptic ulcer disease, gastric cancer) has been mostly demonstrated in geographical areas or ethnic groups with a lower (40–50%) prevalence of *H.pylori* infection in the population. In a situation, where a majority of the population is infected with *H.pylori*, it is reasonable to expect that the putative disease type-specific differences may be indistinguishable on such background. The prevalence of *H.pylori* infection in patients with gastric cancer seems to be underestimated under these circumstances due to an appreciable decrease in the seroprevalence rate in patients with advanced cancer.

The CagA status

CagA is the most studied virulence factor of *H.pylori*. This protein is highly immunogenic, which allows the detection of CagA positive phenotypes by

immunological methods. In the present study, two assays were used to evaluate the CagA status: an immunoblotting (Paper I, V) and CagA-ELISA using a recombinant fragment of the CagA (Paper IV). The latter assay showed good sensitivity and specificity as compared with Western blot (Xiang et al., 1993).

Immunoblotting

In contrast to *H.pylori* seroprevalence, the CagA seropositivity rates as defined by immunoblotting revealed significant differences between the groups studied (Paper I,V). Compared to blood donors, a significantly higher proportion of CagA seropositive subjects was found in patients with gastric cancer ($p=0.008$) and those with non-malignant gastric diseases ($p=0.001$). The highest seropositivity rate was observed in patients with stomach ulcer (91.17%, 31 of 45). Only patients with chronic atrophic gastritis did not differ from the donor's group: 56.1% and 59.0%, respectively. Patients with advanced gastric cancer (stage IV) showed significant decreases in CagA seroprevalence compare to those at stage I+II ($p=0.002$) and patients with peptic ulcer disease ($p<0.001$).

Our findings are in agreement with many other studies showing that the CagA positive phenotype is associated with a more severe pathology including gastric cancer (Blaser et al., 1995; Kuipers et al., 1995b; Parsonnet et al., 1997; Vorobjova et al., 1998; Orsini et al., 1998; Maaroos et al., 1999). At the same time, the cagA status should not be considered as a parameter that could be used to predict the clinical outcome of the infection at the individual level. It was also impossible to discriminate the disease type-specific peculiarities by an immune response to CagA, because a rather high proportion of CagA positive subjects were present in all groups studied, regardless of pathology.

Similarly to the *H.pylori* seroprevalence, a lower proportion of CagA seropositive patients was revealed in patients with gastric cancer and, in addition, in those with atrophic gastritis, possibly due to a decrease in *H.pylori* density colonisation usually observed in atrophic stomach (Siurala et al., 1988; Sipponen, 2002). A clear cut disease stage-dependency was observed in gastric cancer patients with a significant decrease of CagA seroprevalence rate in advanced cancer.

It should be also noted here that in about 30–50% of cases, both CagA-negative and CagA-positive strains or genotypes can be present in one stomach, in one biopsy sample or even in one colony of *H.pylori* culture (Figura et al., 1998). Therefore we believe that, unlike DNA-based techniques, the serology can give more reliable results in CagA status testing because it allows detection of the dominant CagA phenotype in a given individual.

In another study (paper I) we analysed the distribution of 10 major protein bands on immunoblots of H.pylori-positive blood donors (n=23) and patients with gastric cancer (n=72) trying to find the 'cancer-specific' features in the pattern of protein bands. A decline in the recognition of putatively cross-

reacting (33–66 kDa) antigens was noted in the cancer group. The response to the vacuolating toxin-related 85-kDa and CagA 120-kDa protein antigens was not altered but was observed more often in the younger group of cancer patients (under 50 years of age). Some tendency toward a higher proportion of patients infected with CagA-positive strains was observed in patients with intestinal type gastric carcinoma (72% vs 59% for patients with diffuse type of tumor).

We found that about 50% of *H.pylori* ELISA-seronegative patients recognized the 120 kDa (CagA) band as well. This phenomenon has also been described by other investigators (Crabtree et al., 1993; Torres et al., 1998) suggesting that immune response to CagA may be related to past *H.pylori* infection (Ekstrom et al., 2001; Enroth et al., 2001), possibly due to a high immunogenicity of CagA.

IgG Immune response to a recombinant fragment of CagA (Paper IV)

The CagA-ELISA using a recombinant fragment of CagA as antigen showed a good correlation with immunoblotting for CagA-positive and CagA-negative sera with the sensitivity and specificity of 93.5% and 88.5%, respectively. Using O.D. values greater than 0.42 as a cut off limit for strong responders, we found a significantly higher CagA seroprevalence rate in *H.pylori*-seropositive patients with gastric cancer (48.8%) than in the related group of controls (35,4%) (p=0.02). Like with immunoblotting, a significant decrease in the proportion of CagA-seropositive cancer patients was found from disease stage I to stage IV with a significant fall in the response at stage III–IV. No relation to the histological type of tumor (diffuse/intestinal type) was observed.

Immune response to *H.pylori* in relation to ABO(H) and Lewis(a,b) blood group phenotype of the host

The *H.pylori* and CagA seroprevalence in patients with gastroduodenal pathology and blood transfusion donors was correlated with ABO(H), Lewis(a,b) phenotypes and the secretory status (*Se/se*) of the host (Paper II,IV,V). Besides, the strength of IgG immune response to *H.pylori* cell surface proteins as defined by relative antibody activity (RAA) was studied in blood donors depending on their Lewis(a,b) phenotype (Paper II). We also tested whether the immune response to Lewis type 2 Le(x,y) antigens is related to host type 1 Lewis(a,b) histo-blood group phenotype and/or *H.pylori* serologic status of the individual (Paper III).

The *H.pylori* seroprevalence: (Paper II,V)

In blood transfusion donors, no significant differences in *H.pylori* seroprevalence was observed between the individuals of different ABO(H) blood group phenotype: the seropositivity was in the ranges of 82–85%. An insignificantly lower seroprevalence for blood group A and AB individuals was noted among donors (73–79%), patients with gastric cancer (64–71%) and benign gastric disorders (80–81%). However, the Le(a+b-)/non-secretor phenotype group of donors contained a significantly higher proportion of the *H.pylori*-seronegative subjects (34.8%) compared to Le(a-b+)/secretor individuals (14.2%; $p<0.038$) and those with Le negative Le(a-b-) phenotype (8.7%). (Fig.1)

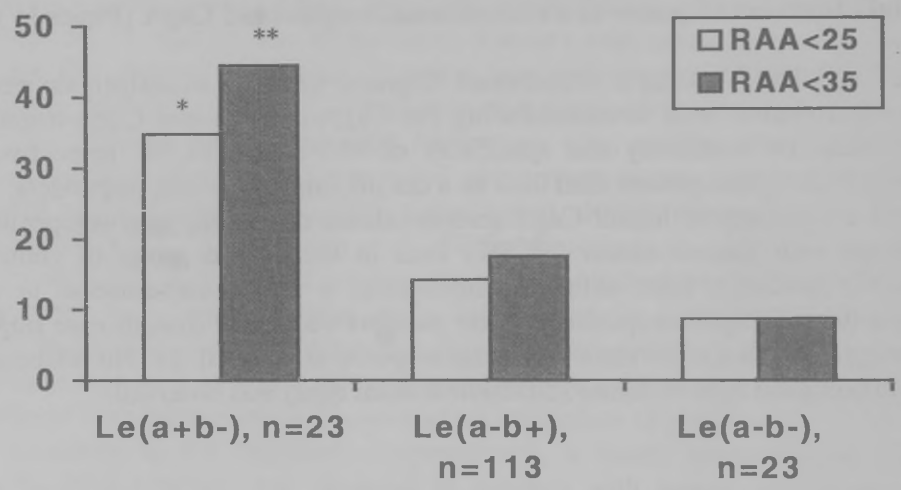

Fig.1. The frequency of H.pylori-seronegative individuals among blood donors with different Le(a,b) blood group phenotypes.

Dark bars, RAA<25 as criterion for H.pylori seronegativity; light bars, RAA<35 (low responders). * – Significantly higher as compared with both Le(a-b+)/secretors and Le(a-b-) group (χ^2=4.26, df 1, P=0.039; OR=3.8, 95%CI 1.1–9.9). ** – Significantly higher as compared with both Le(a-b+)/secretors and Le(a-b-) group (χ^2=6.58, and χ^2 =5.52, df 1, P=0.01; OR=3.8, 95 CI 1.3–11.0 and OR=8.1 95% CI 1.3–46.1, respectively).

Thus it appears that Le (b)-negative individuals are more resistant to *H.pylori* infection. This is in agreement with an idea that Le(b) antigen, which is present in gastric glycoconjugates of secretors, is a receptor for *H.pylori* attachment to gastric mucosa *via* BabA adhesin of *H.pylori* (Boren et al., 1993, 1994; Gerhard et al., 1999). Moreover, the proportion of donors with lower RAA values (low

responders) was significantly higher in Le(a+) than in Le(a-) individuals (P=0.004) with no relation to age. No significant difference was noted between Le(a-b+) and Le(a-b-) groups (P=0.26). These findings suggest that, in addition to Le(b) expression, the presence of Le(a) or some other antigens associated with non-secretor status may be important in the natural resistance to *H.pylori*. One of the candidates may be the T epitope (Galβ1-3 GalANAcα/β) which is known to be expressed on type 3 mucin-type chains of non-secretors, exclusively (Bara et al., 1993) and is further fucosylated in secretors (Okada et al., 1994).

Lower IgG immune response to *H.pylori* in non-secretors indicates that this systemic response seems not to be related to their relative resistance to the infection. One may speculate that these individuals have stronger cell-mediated immune response *in situ*. A more pronounced local inflammation in *H.pylori* infected non-secretors has been demonstrated (Henegan et al., 1998).

The CagA status (Paper IV,V)

With immunoblotting, blood group A donors showed a significantly lower CagA prevalence compared to those of blood group O and B (P=0.0028). (Paper V). Similar differences were observed when individuals possessing blood group A antigen (blood group A and AB) were compared with A antigen-negative (blood group O and B) donors (P=0.006). This was also true for the patients suffering from benign gastric disorders (P=0.04). The difference was less pronounced in patients with gastric cancer.

A significant association of blood group A phenotype with a lower CagA seroprevalence was further demonstrated in blood donors as defined by CagA-ELISA which allows a semiquantitative evaluation of the response (Paper IV). In patients with gastric cancer, a stage-dependent decrease in CagA-seropositivity rate with a drastic fall in the response to CagA at stage IV was observed only in the individuals of blood group O and A.

The donors of Le(a+b-) and Le(a-b-) phenotype revealed a significantly higher proportion of CagA-positive subjects compared to those of Le(a-b+) individuals (P=0.039 and P=0.019, respectively) (IV). As it was described above, the Le(b-) individuals had a lower *H.pylori* seroprevalence. We suggest that higher prevalence of CagA-positive strains in these individuals may be explained by their relative resistance to *H.pylori* infection. It shoud be expected that more resistant individuals could be infected only with a more virulent, i.e. CagA positive, strain.

Some tendency towards a higher proportion of patients infected with CagA-positive strains was observed in patients with intestinal type of gastric carcinoma (72% vs 59% for patients with diffuse type of tumor). There was no Le(a+)-type individuals among patients with gastric cancer. However, the CagA positivity rate in patients of Le(a-b+)/secretor phenotype was very similar to

donors of related phenotype (63.3% and 62.3%, respectively), and was significantly higher than in Le(b-) donors group. As with *H.pylori* seroprevalence, the secretory status had no impact on CagA seroprevalence.

Thus the differences in CagA seroprevalence are more related to the Lewis phenotype of the host rather than the disease-type or clinical outcome of the infection. It seems that the probability of being infected with a CagA positive strain is higher in individuals of Le(b-) phenotype.

The secretory (Se/se) status

The Se/se status, as determined by the secretion of H antigen in saliva, was studied in patients with benign gastric diseases and those with gastric carcinoma.

No significant differences in *H.pylori* and CagA seroprevalence rate was found between secretors and non-secretors in these groups of patients. Higher prevalence of CagA-positive strains (12 of 15, 80%) was found in duodenal ulcer patients who had a significanly higher proportion of non-secretors (15 of 45, 33.3%) compared to patients with chronic gastritis (P=0.008). This is in concordance with a higher prevalence of CagA-positive strains obtained in blood donors of Le(a+)/non-secretor phenotype and described in the previous section. It is to note that there is no strong parallelism between the secretion of H antigen and Le(a,b) phenotype detected by agglutination of erythrocytes with Le antigens-specific antibodies. In particular, about 90% of individuals of Lewis negative phenotype (Le a-b-) are secretors.(Oriol et al., 1992). Besides, some Le(b+) individuals may be so-called 'weak secretors'. It appears that the expression of Lewis antigens on gastric cell glycoconjugates is a more important factor in *H.pylori*-host interplay than the secretion of ABH or Lewis antigens into gastric lumen.

Immune response to Lewis type 2 antigens (Paper III)

An expression of human Le antigens in *H.pylori* as well as an induction of Lewis antigen-specific antibody in infected individuals has been demonstrated by many investigators (Sherburne and Taylor, 1995; Appelmelk et al., 1996; Heneghan et al., 2000). This expression seems to be related to the host Lewis phenotype (Wirth et al., 1997) and important in *H.pylori* adhesion to gastric mucosa (Boren et al., 1993). It has also been considered that this is one of the mechanisms for *H.pylori* to evade the host's immune defence via an antigenic mimicry mechanism since Le antigens are also expressed in normal gastric mucosa cells. We aimed to study whether a level of these antibodies is related to Lewis (a,b) phenotype of the host and *H.pylori* serologic status.

No appreciable differences in IgG immune response to Le(x) epitope between individuals of different Le(a,b) phenotypes were observed when the data were analysed irrespective of *H.pylori* serologic status. Individuals of Le(b+) phenotype revealed a lower proportion of strong responders to Le(y) compared to Le(b-) phenotype subjects. *H.pylori*-seronegative and -seropositive subjects showed different patterns of response. *H.pylori*-seropositive individuals of Le(b-) phenotype showed a significantly higher proportion of strong responders to Le(x) determinant compared to *H.pylori*-seronegative subjects. A similar trend was observed for Le(y) antibody levels. In contrast, Le(b+)/secretors revealed a high natural immune response to these antigens irrespective of *H.pylori* serologic status.

The data suggest that immune response to Lewis type 2 determinants is related to both the *H.pylori* serologic status and the Le(a,b) phenotype of the host. Higher immune response to Le(x) in *H.pylori*-infected individuals observed in this study may be related to the findings of Byrd et al. (1997) who reported on an aberrant expression of Le(x) in the surface gastric epithelium of *H.pylori*-infected individuals. On the other hand, Kuipers et al. (1997) showed that infected subjects with low levels of Le(x) antibodies had a higher risk of gastric atrophy. However, both groups of authors did not relate their findings to the Lewis phenotype of the host. One might speculate that putatively 'immune' Le(x) antibodies which appear in response to *H.pylori* in Le(b-) type subjects may be more pathogenic in terms of their ability to induce inflammation. It remains unclear, however, to what extent a natural or *H.pylori*-induced immune response to Lewis type 2 antigens might be beneficial or detrimental for the host and how it is related to the clinical outcome of the infection.

H.pylori infection and humoral immune response to the tumor-associated Thomsen-Friedenreich antigen (T Ag) (Paper VI,VII)

The Thomsen-Friedenreich (T) antigen (Galβ1,3GalNAcα/β-O-Ser/Thr) is expressed in a majority of human carcinomas and related to invasion, metastases and prognosis (Springer, 1984). Natural T-specific antibodies are present in the blood of every individual. Their level has been shown to be low in serum of patients with different malignancies (Springer, 1984, Desai et al., 1995) including gastric cancer (Kurtenkov et al., 1999), and in gastric premalignant conditions (Smorodin et al., 1997). Since the *H.pylori* infection is very closely related to gastroduodenal pathology (Kuipers, 1999; Kuipers et al., 1995, McGee and Mobley, 1999) we suggested that one of the mechanisms might be associated with *H.pylori* infection. We tested the hypothesis that *H.pylori* infection may alter natural immune response to tumor-associated

Thomsen-Friedenreich (T) antigen, thus modulating natural immune mechanisms against cancer.

The patients with gastric cancer (n=186), benign gastric diseases (n=126) and 186 blood transfusion donors were tested for blood level of T antigen specific IgG and IgM antibodies. The data were correlated with *H.pylori* serologic status, disease type, *Se/se* status, stage of cancer, ABH and Lewis (a,b) blood group phenotype of the host.

The changes in T antibody levels were analysed in two ways: by comparison (i) between the *H.pylori*-seronegative and seropositive subgroups within each group (donors, patients) to look for a relation of the changes to the *H.pylori* infection, and (ii) between the groups for both infected and noninfected individuals trying to find putative disease type-specific features.

An impact of *H.pylori* serologic status

The changes in IgM and IgG T-antibody level in relation to the *H.pylori* serologic status of patients and blood donors are shown in Fig.2. A common feature for IgG immune response was that the *H.pylori*-seropositive group showed a higher proportion of patients with high levels of T antibody (strong responders) compared with the related *H.pylori*-seronegative subgroups. This difference was observed in all groups of patients (gastric cancer, peptic ulcer, chronic gastritis) and blood donors reaching the statistical significance in patients with gastric cancer and gastritis.

In *H.pylori*-seropositive blood donors, the level of IgG T-specific antibodies (O.D. values) positively correlated with anti-*H.pylori* IgG anibody level (RAA values) (VII). It has been shown that higher density of antral gastric mucosa colonization with *H.pylori* is associated with a higher IgG immune response to the organism (Kreuning et al., 1994; Hsu et al., 1997) and stronger local inflammatory response to *H.pylori* (Henegan et al., 1998, 2000). We suggest that the above mentioned correlation may reflect the density of *H.pylori* colonization or the degree of T epitope expression in a given *H.pylori* strain. No correlation was observed in patients with cancer possibly due to a significant decrease of T specific antibody levels in a majority of patients (Kurtenkov et al., 1999). Unlike IgG antibody, the IgM response was not closely related to *H.pylori* serology. Compared to blood donors, a dramatic decrease of IgM T antibody level in every group of patients was found irrespective of *H.pylori* status, especially in Le(a-b+) type patients with gastric cancer.

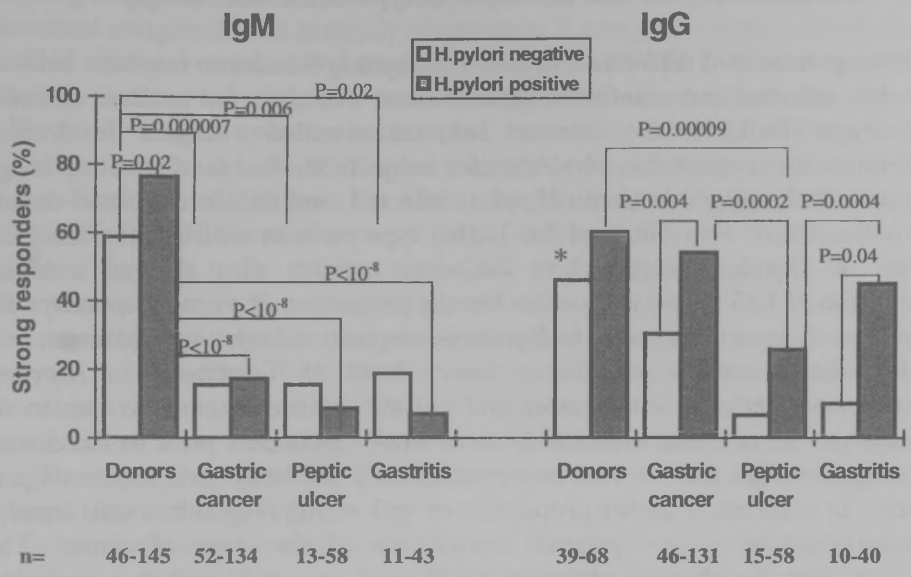

Fig.2. The proportion of IgM and IgG strong responders to T-epitope in blood donors and patients by H.pylori serology.

P values are shown for significant differences as calculated by Fisher's exact test.
* Significantly higher compared with H.pylori – seronegative group of patients with peptic ulcer (P=0.005) and gastritis (p=0.04).
n- the number of individuals for H.pylori-seronegative and H.pylori-seropositive groups respectively.

Interestingly, some disease type-specific traits in T response may also be seen. The *H.pylori*-seropositive patients with gastric cancer and gastritis showed an IgG response similar to that of donors. At the same time, the *H.pylori*-seropositive patients with peptic ulcer disease revealed only slight increases in IgG response: a proportion of strong responders was significantly lower than in any other *H.pylori*-seropositive group regardless of their *H.pylori* status.

The data suggest that *H.pylori* infection stimulates the IgG immune response to T epitope. Our preliminary data about the decrease of immune response to T epitope in some patients after *H.pylori* eradication (VI) further support the idea that an increase in T antibody level is related to *H.pylori* infection. The effect seems to be dependent on the initial level of T antibody and was observed in subjects who were obviously low responders before being infected with *H.pylori* and enhanced their T-specific immune response due to the infection.

Relation to ABH and Lewis phenotype of the host (Paper VI)

A more pronounced difference in anti-T antigen IgG immune response between *H.pylori*-infected and uninfected blood donors was observed in those of Le(b-) phenotype (P=0.04): the infected subjects revealed a higher level of T antibodies. In contrast, Le(a-b+)/secretor subjects showed no distinction in IgG response to T antigen between *H.pylori*-infected and uninfected blood donors. A similar trend was obtained for Le(b-) type patients with gastric cancer. A group of *H.pylori*- seropositive Le(a-b+) patients also showed a higher proportion of IgG strong responders but the proportion of strong responders was significantly lower compared to *H.pylori*-seropositive Le(b-) type patients.

It remains unclear whether a lower level of T-antibody in *H.pylori*-seronegative patients with cancer and chronic gastric diseases is due to the disease *per se* or these individuals were weak responders prior to the disease development. We did not find any relation of T antibody level to the stage of cancer. In contrast, a higher proportion of IgG strong responders was found in *H.pylori*-infected cancer patients irrespective of the stage of cancer. This suggests that the changes observed should not be considered as a secondary tumor-induced event. Since the level and profile of natural antibodies is reported to be fairly stable for years (Springer et al., 1984; Kaveri et al., 1998) we speculated that there is some kind of enrichment of weak T responders among individuals which are predisposed to gastric pathology or suffering from chronic gastric diseases including cancer. An association of T-antibody level with Le(a,b) histo-blood group phenotype suggests a possible genetic background.

Thus, *H.pylori* infection is associated with an increased IgG immune response to tumor-associated T antigen. This systemic impact is dependent on the Lewis phenotype of the host and in part disease-type specific. These findings suggest that *H.pylori* may be indirectly involved in gastric carcinogenesis *via* modulation of natural cancer-related immune mechanisms.

Expression of tumor-associated Thomsen-Friedenreich antigen in *H.pylori* (Paper VI, VII)

A striking similarity in the increase of IgG T-response in different groups of *H.pylori*-infected individuals compared to uninfected ones (Fig.2) suggests the existence of a common pathway. We have suggested that one of the mechanisms might be an expression of carbohydrate Thomsen-Friedenreich (T) antigen (Galβ1,3GalNAcα/β-O-Ser/Thr) in *H.pylori*, like it has been shown for many carbohydrate epitopes on bacteria (Blackwell, 1989). If this is the case, an

ongoing antigenic stimulation in *H.pylori*-infected subjects may lead to an increased antigen-driven antibody response to T antigen in some individuals.

Cell surface membrane extracts of *H.pylori* NCTC 11637 strain and 13 clinical isolates of *H.pylori* were analysed by immunoblotting with five different T antigen-specific human and murine monoclonal antibodies. Two major protein bands of ~68kDa and 58 kDa were immunostained on blots of *H.pylori* extract with T specific Mabs but not immunostained with unrelated MAb (Fig.3). However, extracts from different *H.pylori* strains showed different patterns in the relative expression of T antigen-related bands. The 68 kDa protein associated T antigen expression was higher in patients with more severe pathology (gastric cancer, peptic ulcer disease) compared to strains derived from patients with chronic gastritis. This protein band was almost absent in extracts from the NCTC 11637 *H.pylori* standard strain. Besides, this strain did not show reactivity with MAb 9H8 which was reported to be specific for T antigen, however, the peptide core seems to be also involved in the epitope (Sikut et al., 1996)

In blocking experiments, the PNA and T-specific rabbit antiserum almost completely abolished the binding of human TF1, 5C7 and murine JAA-F11 MAb. The binding of T specific MAb to the 58 kDa protein was also blocked by rabbit antiserum against heat shock proteins of *H.pylori*. Both T-related bands did not bind the anti-H-type-2 mouse IgM MAb.

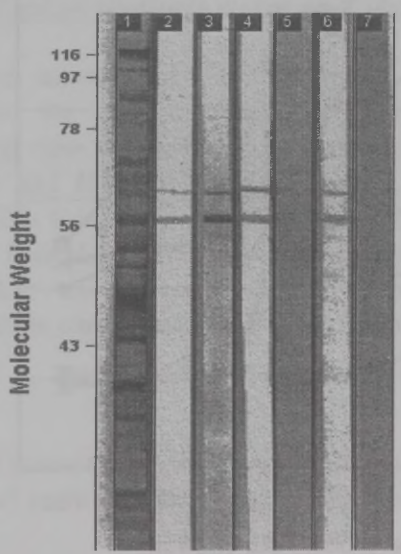

Fig.3. Western blot analysis of T antigen-specific monoclonal antibodies on cell surface membrane extract from H.pylori NCTC 11637 strain.

Lane 1, H.pylori seropositive human serum (RAA - 94); Lane 2, human Mab 5C7; Lane 3, human Mab TF1; Lane 4, murine Mab 3C9; Lane 5, murine Mab 9H8; Lane 6, murine Mab JAA-F11; Lane 7, murine Mab 18OLE (anti-H type 2). Molecular mass markers (kDa) are at left.

In cell-ELISA, binding of TF1 and 5C7 human MAb to *H.pylori* cells was noted, as was binding of JAA-F11 and 9H8 MAb (Fig.4). All T-specific MAbs reacted in a dose-dependent manner. T-epitope unrelated anti-leukosialin MAb (3A1) showed very weak nonspecific binding irrespective of the dilution.

A positive correlation was found between the level of serum IgG antibodies to T antigen and anti-*H.pylori* antibodies (RAA values) in *H.pylori*-infected blood donors (r=0.57, P>0.001). In contrast, no such correlation was observed in *H.pylori*-seronegative blood donors and patients with gastric cancer, irrespective of *H.pylori* status.

Thus, the cancer-related autoimmunogenic T epitope is expressed in surface membrane glycoconjugates of *H.pylori* and associated with an alteration of the natural immune response to T antigen in infected subjects.

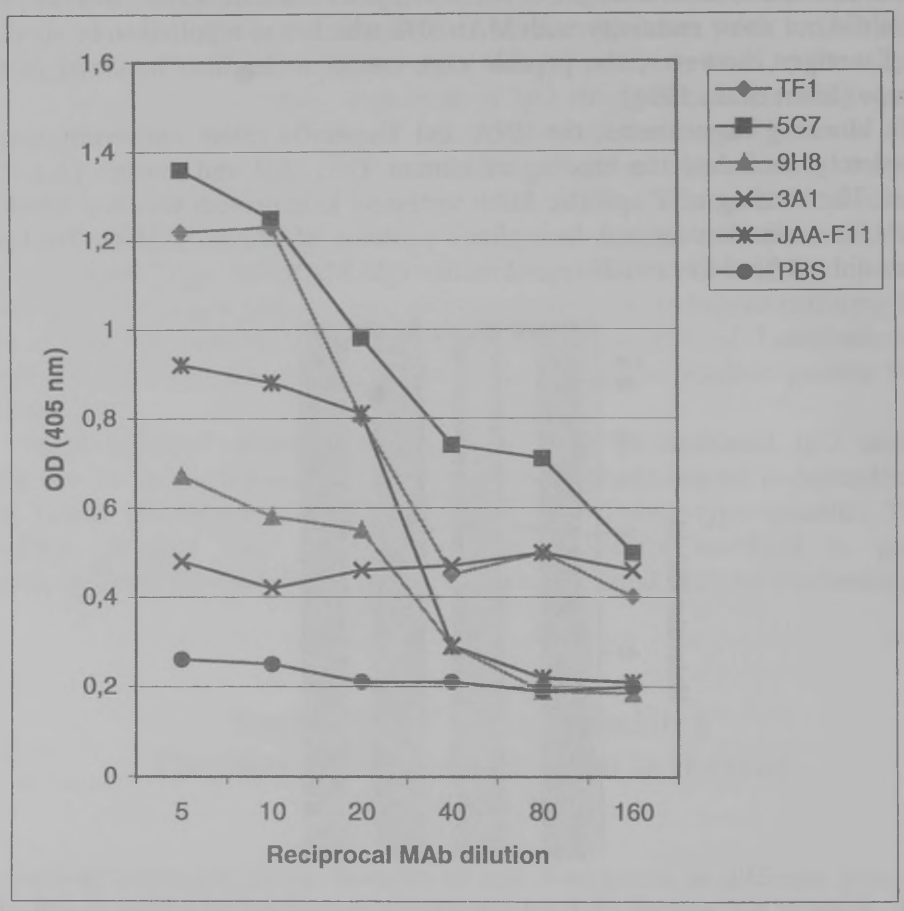

Figure 4. Binding of T antigen-specific monoclonal antibodies to H.pylori NCTC 11637 strain cells as assessed by cell-ELISA with 108 bacteria. OD (405 nm), optical density at 405 nm.

H.pylori infection and survival of patients with gastric carcinoma: relation to the level of T antigen specific antibodies (Paper VIII)

Our findings that H.pylori infection modulates humoral immune response to tumor-associated T antigen as well as the evidence that H.pylori itself expresses this epitope prompted us to study the possible clinical relevance of these phenomena.

It has been shown that an increase in the level of antibodies against T and Tn epitopes after active immunotherapy of cancer with mucin-type vaccines containing these epitopes was associated with a more favourable prognosis of patients with cancer (MacLean et al., 1992; Livingstone et al., 1997). On the other hand, low level of T-specific antibodies is associated with an increased risk for cancer (Springer, 1984). The expression of T antigen in cancer cells is related to the biological behaviour of tumors and the prognosis of cancer patients (Springer, 1984; Takanami, 1999; Baldus and Hanish, 2000). Thus, we proceeded from the assumption that higher levels of T specific antibodies induced by *H.pylori* infection may be beneficial for the patients with cancer due to the anti-tumor potential of these antibodies.

H.pylori serologic status and survival

A significantly better survival rate was observed in *H.pylori* seropositive patients at stage I of the disease compared to *H.pylori*-seronegative ones (Fig.5A). The survival time (median±SE) was 60.0±3.8 and 37.0±7.8 months for *H.pylori* positive and *H.pylori*-negative patients, respectively (P=0.0004, log-rank test). A similar trend was noted for the patients at stage II (Fig.5 B): 56±2.7 and 46±11.3, P=0.064. No difference in survival was noted for patients with advanced gastric cancer *(unpublished)*. Thus, *H.pylori*-seropositive patients with early gastric cancer had better survival rates compared to *H.pylori*-seronegative patients.

Humoral immune response to T antigen and survival of patients with early gastric cancer

A comparison of patient's survival in relation to anti-T Ag antibody levels, calculated irrespective of *H.pylori* status, showed that stage I patients with higher level of T Ag specific IgG antibody had significantly better survival rates compared to those with low levels of antibody (weak responders): median survival time 62.0±2.1 and 35±1.4 months, respectively; P=0.0001 (Fig.5B).

A significant over-representation of patients with high level of anti-T antigen IgG antibodies (strong IgG responders) was observed among *H.pylori*-seropositive stage I patients compared to *H.pylori*-seronegative ones (P=0.023, Fishers exact test). A similar trend was found in patients at stage II, but the differences did not reach statistical significance (P=0.13). The same was true for IgM T antibody level (P=0.12).

To clarify whether better survival of *H.pylori*-seropositive patients is related to a higher level of anti-T antibody in infected patients, the comparisons were made for strong and weak responders in both *H.pylori*-seropositive and *H.pylori*-seronegative subgroups of patients. In fact, only *H.pylori*-seropositive strong IgG responders were better survivors (P=0.0001), (Fig 5C) whereas strong IgG responders in the *H.pylori*-seronegative group were not (P=0.96). This suggests that the presence of *H.pylori* infection seems to be an important independent prognostic factor. Similar trends were observed in a group of patients at stage II.

It was a general regularity that the level of IgM T antigen-specific antibodies did not correlate with survival. One of the reasons is that a very low level of natural IgM T antibody, already at early stages of the disease, is a characteristic of patients with various cancers (Springer, 1984; Kurtenkov et al., 1999). One may speculate that acquired IgG immune response to T antigen induced by *H.pylori* is a more potent mechanism of eliminating tumor cells in the circulation by antibody-dependent effector mechanisms. The fact that no association of T antibody level or *H.pylori* serologic status with survival was noted for patients with advanced gastric cancer (unpublished) suggests that this mechanism may be effective for the elimination of only small numbers of tumor cells or micrometastasis which are present or appear in the circulation after surgery, thus preventing metastasis and improving the survival.

In addition to antibody-dependent reactions, other immunological mechanisms might be considered to explain an association between *H.pylori* infection and better survival rates of patients with early gastric cancer. *H.pylori* upregulates the proinflammatory cytokines IL-6, IL-8, TNFα, IFNγ that have anti-tumor or growth inhibiting potential, an inhibitory effect on angiogenesis or stimulate differentiation (Kang et al., 1999; Arany et al., 1999; Jenkinson et al., 2002). Contact of lymphocytes with *H.pylori* was reported to stimulate the production of IFN-γ and natural killer cell activity (Tarkkanen et al., 1993). Theoretically, the *H.pylori* glycosidases and/or glycosyltransferases may create the tumor-related epitopes on host gastric glycoconjugates or tumor cells like it has been shown, for instance, for tumor-associated alphaGal-epitope expression induced by *Klebsiella pneumonia* enzymes on erythrocytes (Hamadeh et al., 1996), with the following induction of autoimmune response.

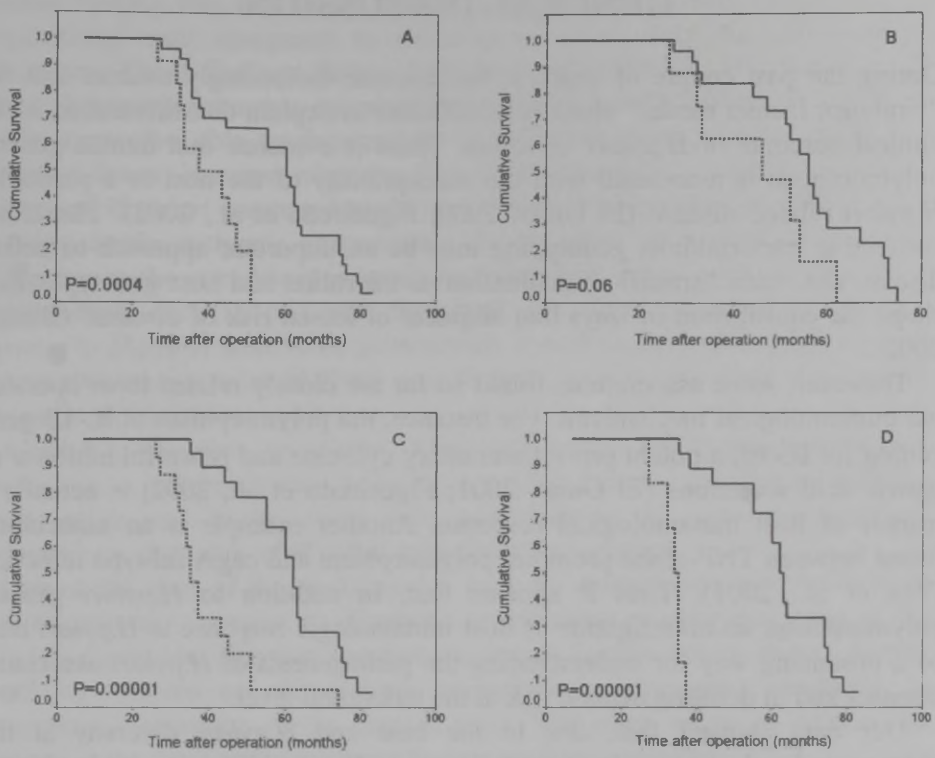

Fig.5. Cumulative survival plots (Kaplan-Meier) of gastric cancer patients in relation to H.pylori serologic status and/or anti-T antigen antibody level.

A – stage I gastric cancer patients (n=44), dark line – H.pylori-seropositive patients; dotted line – H.pylori-seronegative patients. B – the same for stage II gastric cancer patients (n=43).

C – stage I gastric cancer patients (n=37), dark line – patients with high level of anti-T antigen IgG antibodies (strong responders); dotted line – patients with low level of anti-T antigen IgG antibodies (weak responders). The cumulative survival is calculated irrespective of H.pylori serologic status.

D – H.pylori-seropositive gastric cancer patients at stage I (n=26); dark line – strong responders, dotted line – weak responders.

P values as calculated by log-rank test.

In summary, *H.pylori*-seropositive patients with early gastric cancer have better survival rates than *H.pylori*-seronegative patients, and this impact is related to the up-regulation of the T antigen specific immune response in *H.pylori*-infected individuals.

GENERAL DISCUSSION

During the past couple of years, it has become increasingly evident that the "virulence factors model" alone is insufficient to explain the differences in the clinical outcome of *H.pylori* infection. There is evidence that human genetic polymorphism is associated with the susceptibility of the host to a particular *H.pylori*-related disease (El Omar, 2000; Figueiredo et al., 2002). Therefore, combining bacterial/host genotyping may be an important approach to define disease risk since "specific combination of microbial and host genotypes may shape the equilibrium in ways that augment or lessen risk of disease" (Blaser, 2002).

However, some associations found so far are closely related to or operated *via* immunological mechanisms. For instance, the polymorphism of IL-1B gene coding for IL-1β, a potent pro-inflammatory cytokine and powerful inhibitor of gastric acid secretions (El Omar, 2001; Figueiredo et al., 2002) is actually a marker of host immunological response. Another example is an association found between TNF-alpha promoter polymorphism and cagA subtype infection (Yea et al., 2001). Thus it appears that, in addition to *H.pylori* genetic polymorphism, an investigation of host immunologic response to *H.pylori* may be a promising way for understanding the pathogenesis of *H.pylori*-associated diseases and in defining disease risk at the individual level.

Our data showed that, due to the host and *H.pylori* diversity at the phenotypic level, the host immune response to *H.pylori* infection is also highly polymorphic in both its strength and profile. In spite of the rather small part of host phenotypic traits studied, i.e. ABH and Lewis blood group phenotype and *Se/se* status, we found that immune response to both glycine cell surface extract of *H.pylori* and to a recombinant fragment of the CagA showed an association with ABH and Lewis phenotype of the host.

A higher proportion of *H.pylori*-seronegative individuals was found among blood donors of Le(a+)/non-secretor phenotype, suggesting that the Lewis histo-blood group antigens are implicated in the mechanisms of naturally occuring resistance to *H.pylori* infection. Interestingly, the proportion of *H.pylori*-seronegative individuals among blood donors was strikingly similar to that of Le(a+)/non-secretors in Estonia, i.e. 16.4% and 11.7% (Mark et al., 1994), respectively. Moreover, there was some disposition for individuals of Le(b-) phenotype to be preferentially infected with CagA positive strains of *H.pylori*. An association of CagA-positive strains with a more severe pathology demonstrated by many investigators may be related to our findings because a significantly higher proportion of non-secretors, which are mostly of Le(b-) phenotype subjects, was found among patients with duodenal ulcer. It seems that these associations are not related to the BabA or SabA adhesin of *H.pylori* (Boren et al., 1993; Mahdavi et al., 2002) because they were observed in Le(b-) type individuals. This suggests that other mechanisms may be involved.

Another finding was that blood group A individuals showed a lower CagA seropositivity rate compared to subjects of other ABO(H) blood group phenotypes. These findings further support the idea that the polymorphism of gastric glycoconjugates may appreciably influence the *H.pylori*-host interplay.

We were also able to demonstrate that individuals of Le(a+) phenotype showed a lower immune response (RAA values) to cell surface membrane antigens of *H.pylori* compared to those of other Lewis phenotypes. Thus, even by using crude antigenic preparations it was possible to demonstrate the differences in the strength of immune response between individuals of various Le phenotype. ABO(H) or Lewis phenotype-associated differences in immune response to *H.pylori* were more pronounced if well characterized antigens such as recombinant fragment of CagA and T disaccharide were used as antigens. This point will be discussed below.

Altogether the data show that ABH and Lewis blood group phenotype distribution in the population should be considered as an important modulating factor which has to be taken into consideration in immunological and epidemiological studies of *H.pylori*-host interrelationships.

One of the aims of the study was to look for disease-type specific traits in humoral immune response to *H.pylori*. At present it is clear that none of the currently identified *H.pylori* putative virulence factors shows strong disease specificity. Moreover, they show remarkable geographic variability in prevalence irrespective of the disease type. The same seems to be true for various events associated with these factors such as immunity.

In the present study, an immune response to *H.pylori* antigens was also shown to be not strongly related to the type of the disease if comparisons were made irrespective of host blood group polymorphism. An ability of the host to elicit immune response to *H.pylori* was fairly variable and in part dependent on blood group antigens polymorphism. However, some statistically significant associations were demonstrated. Like many other investigators, we found a significantly higher CagA seroprevalence among patients with gastric cancer and peptic ulcer disease (V). A higher proportion of non-secretors was found in patients with duodenal ulcer. Compared to donors, the cancer patients showed a decline of the response to low molecular weight (<28,33–66kDa) *H.pylori* antigens (I).

It has been shown that only 6–7 % of *H.pylori* genes may vary between the strains (Alm et al., 1999) suggesting that no appreciable difference in the spectrum of immune response to peptide-type antigens should be expected between the strains. However, if possible post translational modifications such as glycoforms are included, the heterogeneity of the strains would be greatly increased. Interstrain variations in the expression of T glycotope-related proteins in *H.pylori* and some disease-type differences in immune response to T epitope may be related to this issue. Patients with peptic ulcer disease showed very low level of T antigen specific IgG antibodies compared to other groups studied. In contrast, seropositive patients with gastric cancer and those with

chronic gastritis revealed significantly higher T antigen specific IgG response compared to seronegative subgroups. However, in immune response to *H.pylori*, variations that are related to blood group phenotype of the host are dominant over disease-specific changes in the response. The difference became more visible for well characterized antigens such as T disaccharide.

Several arguments were taken into account for using the Thomsen-Friedenreich (T) antigen as a model to test the hypothesis of whether H.pylori infection might influence natural immune mechanisms against cancer: (i) the tumor-associated T epitope, like many other carbohydrate antigens, was shown to be expressed in tumor cells and some microorganisms (Springer, 1984; Blackwell, 1989) (ii) theT epitope is present as a cryptic antigen in gastric mucin-type (type 3) glycoconjugates of secretors (Okada et al., 1994); (iii) it is expressed in non-secretors (Bara et al., 1993) and thus its expression is related to Le phenotype of the host;.(iv) natural humoral immune response to this antigen was demonstrated to be low in patients with gastric cancer and premalignant conditions of the stomach (Smorodin et al., 1997). And finally, a level of naturally occuring T antigen specific antibodies is also related to Le (a,b) phenotype (Kurtenkov et al., 1999).

It has been shown recently that *H.pylori* is very closely associated with extracellular MUC5AC mucin and epithelial cells that produce MUC5AC suggesting a role of this mucin in the adhesion of *H.pylori* to the gastric mucosa (Van den Brink et al., 2001). *H.pylori* strains that express the BabA adhesins were shown to bind to the Le(b)-positive glycoforms of MUC5AC mucin in individuals expressing the Le(b) antigen (Linden et al., 2002). We suggest that the lower immune response to *H.pylori* we found in Le(a+) individuals may be related to a lower density of *H.pylori* due to a weaker *H.pylori* adhesion to gastric epithelial cells compared to subjects of Le(b+) phenotype.

In this study, we demonstrated that *H.pylori* serologic status is an important factor that influences natural immune response to T epitope. A clear-cut difference in anti-T antigen IgG antibody level was observed if individuals were divided according to *H.pylori* status: *H.pylori*-seropositive patients with gastric cancer and chronic gastritis showed a significantly higher immune response to T antigen compared to seronegative subgroups. A similar trend was found in patients with peptic ulcer disease though a very low T antibody level was a characteristic of these patients. Moreover, a level of IgG T-specific antibodies in serum of blood donors positively correlated with anti-*H.pylori* IgG antibody level which has been shown to be related to the density of *H.pylori* colonization (Pronovost et al., 1994; Kreuning et al., 1994; Hsu et al., 1997). In addition, an ability of the host to elicit an acquired IgG immune response to the putatively *H.pylori*-derived T epitope was associated with the Lewis phenotype of the host and was more pronounced in individuals of Le(b-) phenotype. Preliminary data about the decrease of immune responses to T antigen in some patients after *H.pylori* eradication further support the idea that an increase in T antibody level is related to *H.pylori* infection. A modulation of naturally occuring immune

response to tumor-associated T glycotope in *H.pylori*-infected individuals suggests that *H.pylori* infection may alter natural immune mechanisms against cancer. However, the degree of this modulation varied appreciably between individuals and could not be explained by only the blood group phenotype polymorphism.

Further, we tested the hypothesis that *H.pylori* itself may express cancer-related T antigen and presented immunological evidence that T antigen is expressed in at least two surface membrane glycoconjugates of *H.pylori* with molecular mass of ~58kDa and 68kDa. The specificity of T antigen specific MAbs reaction with *H.pylori* was shown in that immunostaining was blocked with PNA and rabbit antiserum to T antigen. Moreover, different *H.pylori* strains seem to express the T epitope to a different degree with some relation to a particular gastric pathology. Variations in T antigen expression between the clinical isolates of *H.pylori* suggest that this antigen may be of value for further characterization of *H.pylori* diversity. An immunological approach allows for better characterization of alterations in the glycosylation of *H.pylori* proteins which are still difficult to evaluate by other means.

To evaluate possible clinical relevance of *H.pylori* infection-associated modulation of humoral immune response to T antigen, we analysed the survival time of patients with early gastric cancer depending on their *H.pylori* serologic status and the level of T antigen-specific antibodies in the blood before surgery. Highly significant ($p<0.00001$, log-rank test) association of better survival with both *H.pylori*-seropositive status of patients and a level of T specific IgG antibody was demonstrated (Fig. 5). However, the difference in survival between the patients with high and low level of T antigen IgG antibody was found only for *H.pylori*-seropositive individuals. This indicates that the presence of *H.pylori* infection is an important independent prognostic factor. No significant correlation with prognosis was found for IgM T antigen-specific antibodies. One may speculate that acquired IgG immune response to T antigen induced by *H.pylori* is a more potent effector mehanism to eliminate tumor cells in the circulation by antibody-dependent effector mechnisms, and to interfere with metastasis than natural low affinity IgM T-specific antibodies. Besides, very low level of IgM isotype T antibodies, already at the early stages of the disease, is a characteristic of patients with various cancers (Springer, 1984; Kurtenkov et al., 1999).

The main associations between humoral immune response to *H.pylori* and blood group related antigens as well as their possible relation to the pathogenesis of *H.pylori* infection and the clinical relevance are summarized in Table 1.

Table 1. Associations found between IgG immune response to *H.pylori* and blood group related antigens

Antigens	Association	Comment
ABO(H)	Blood group A phenotype is associated with a significantly lower seroprevalence of CagA positive *H.pylori* strains in blood donors and patients with benign gastric diseases but not in patients with gastric cancer (V).	This should be taken into consideration in epidemiological and immunological studies of *H.pylori*-host interplay in populations with different distribution of ABH blood group phenotypes.
	Gastric cancer patients of blood group O phenotype have a higher proportion of CagA-seropositive individuals at early stages of disease (IV).	This may be related to a higher degree of inflammation in blood group O individuls (Alkout et al.,2000) and to a more rapid progression of gastritis to atrophy and cancer.
Lewis antigens	*H.pylor*i seroprevalence is significantly lower among individuals of Le(b-) phenotype. Lower IgG immune response to *H.pylor*i was observed in Le(a+b-) /non-secretor individuals (II).	Individuals of Le(b-) phenotype are more resistant to *H.pylori* infection, possibly due to the absence Le(b) antigen which is a receptor for BabA adhesin of *H.pylori*.
	Lewis(b-) phenotype is associated with a higher prevalence of more virulent CagA-positive strain of *H.pylori* (V).	Le(b-) type individuals may be infected mostly with more virulent strains, because of their higher resistance to *H.pylori* infection.
	Increased IgG immune reponse to T antigen in *H.pylori* infected blood donors and gastric cancer patients was predominantly observed in individuals of Le(b-) phenotype. In addition, *H.pylori* seropositive Le(b-) individuals showed higher proportion of strong responders to Le(x) (III, VI).	H.pylori seropositive Le(b-) individuals with high level of T antigen specific antibody are potentially at lower risk for cancer development? In contrast, they may be proned to autoimmunity?

Antigens	Association	Comment
	H.pylori infection is associated with an enhanced IgG immune response to T antigen in patients with gastric cancer and non-malignant gastric diseases (VI).	This suggests that *H.pylori* infection may modulate natural immunological mechanisms against cancer
Blood-group related tumor-associated Thomsen-Friedenreich (T) antigen	Immunological evidence is presented that tumor-associated T antigen is expressed in *H.pylori*, and this expression varies between the strains (VII).	T antigen expression in *H.pylori* may be of value for further characterization of *H.pylori* diversity. Higher expression of 68 kDa T antigen-related glycoprotein is associated with more severe pathology (cancer, duodenal ulcer).
	H.pylori seropositive patients with early gastric cancer are better survivors than *H.pylori* seronegative patients. This association was mostly pronounced in patients with high level of T antigen specific IgG antibodies (VIII).	This implies that both *H.pylori* serologic status and the level of T antigen specific antibodies may be used as an additional prognostic criteria for surgically treated patients with early gastric cancer.

A growing body of evidence suggests that *H.pylori* infection may actually have some beneficial effects. The carriage of the more virulent CagA-positive strain was shown to be associated with a reduced risk of esophageal and gastric cardial adenocarcinoma and of gastroesophageal reflux disease (Chow et al., 1998; Kuipers,1999; Loffeld et al., 2000a). *H.pylori* infection may protect against diarrheagenic gastrointestinal infections in children (Rothenbacher et al., 2000). Our data showed better survival of *H.pylori*-infected patients with gastric cancer suggesting that this infection may be beneficial for patients with cancer. Therefore, eradication of *H.pylori* becomes more controversial, especially in individuals of older age.

A key feature of our findings is an association of *H.pylori* infection with an increased immune response to tumor-related T epitope and better survival of *H.pylori*-infected patients with gastric carcinoma, though there is no evidence so far for a direct cause-and-effect relationship between the expression of T antigen in *H.pylori* and a higher level of T antibody. We are aware that these data are the first evidence of the kind and should be further confirmed by other investigators. Anyway, a significant association of T antibody level with *H.pylori* infection and the survival of *H.pylori*-infected cancer patients strongly indicates on the pathogenetic role of *H.pylori* and these antibodies in protective immunity against cancer. It remains to be elucidated, however, whether the systemic impact of *H.pylori* infection on the immune response to tumor-related T antigen may work in concert with or contrary to the other *in situ* operating

mechanisms which are known to be involved in the pathogenesis of chronic gastric diseases including cancer. This could in part explain why only a small minority of *H.pylori*-infected individuals develop gastric cancer. We speculate that, if other things being equal, the *H.pylori*-infected individuals with high levels of T antigen specific antibodies might have some protection against gastric cancer development. Anyhow, since infection with *H.pylori* is relatively common, it provides a unique model to study the impact of infections on anti-tumor immunological mechanisms. *H.pylori* infection is treatable and so the model can be easily manipulated. And finally, this model may be used to further demonstrate that, in addition to microbial diversity, several host-dependent factors may alter host-microbe immunological interplay and influence the clinical outcome of the infection.

CONCLUSIONS

1. In the Northen Estonia, a high proportion of *H.pylori*-seropositive individuals (75.5–86.2%) was demonstrated among patients with gastroduodenal pathology and in age-related blood transfusion donors group with no association with a particular disease. In patients with gastric cancer, a stage-dependent decrease in the seropositivity rate was shown. As defined by immunoblotting, a high incidence of more virulent CagA positive strains of *H.pylori* (74.2–91.2%) was characteristic of patients with gastroduodenal pathology except those with atrophic gastritis (56.1%) and advanced gastric cancer (62.5%). The highest proportion of CagA-seropositive individuals was found among patients with duodenal (81.3%) and gastric (91.2%) peptic ulcer disease. A decline in the recognition of putatively cross-reacting (33–66 kDa) antigens was noted in cancer patients.
2. An immune response to cell surface membrane antigens of *H.pylori* NCTC 11637 strain was strongly influenced by ABH and Lewis(a,b) phenotype of the host. This impact had more common rather disease-type specific traits:
   - Blood transfusion donors of Le(a+b-)/nonsecretor phenotype revealed a significantly higher proportion of *H.pylori*-seronegative subjects and a lower IgG immune response to *H.pylori* compared with the individuals of Le(a-b+)/secretor phenotype.
   - As determined by both immunoblotting and enzyme-linked immunosorbent assay with a recombinant fragment of the CagA, blood group A blood transfusion donors and patients with non-malignant gastric diseases showed a significantly lower CagA seropositivity rate compared to subjects of other ABO(H) blood group phenotypes. This association was less pronounced in patients with gastric cancer who showed a significant stage-dependent suppression of immune response to the CagA with no relation to age, gender or tumor histology.
   - A significantly higher CagA-seroprevalence was demonstrated among blood donors of Le(b-) blood group phenotype compared to those of Le(b+) phenotype. This indicates that Le(b-) subjects are predisposed to be infected with CagA-positive strains of *H.pylori*. The secretory (*Se/se*) status of patients with chronic gastric diseases was not associated with CagA serologic status though a significantly higher proportion of non-secretors was found among patients with duodenal ulcer.
3. IgG immune response to Lewis type 2 determinants [Le(x), Le(y)] is related to both the *H.pylori* serologic status and Le(a,b) phenotype of the host. Individuals of the Le(b+)/secretor phenotype revealed a stronger immune response to Le(x) and Le(y) epitopes irrespective of *H.pylori* serologic status. In contrast, *H.pylori*-infected Le(b-) type individuals showed higher

proportion of strong responders to Le(x) antigen compared with the *H.pylori*-uninfected subgroup.

4. *H.pylori* infection is associated with an increased immune response to cancer-associated Thomsen-Friedenreich (T) antigen (Galβ1,3-GalNAcα/β-O-Ser/Thr). This systemic impact appears to be dependent on Lewis(a,b) phenotype of the host and in part disease-type specific. Thus, *H.pylori* infection may be indirectly involved in gastric cancerogenesis *via* modulation of natural immune mechanisms against cancer.
5. An immunological evidence was presented that the cancer-related autoimmunogenic T epitope is expressed in *H.pylori* and this expression is associated with an alteration of natural immune response to T antigen in infected subjects. Different *H.pylori* strains seem to express T epitope to a different degree with some relation to a particular gastric pathology.
6. The survival of patients with early gastric cancer is significantly better in *H.pylori*-seropositive patients, and this phenomenon is related to up-regulation of T antigen-specific IgG immune response in *H.pylori*-infected individuals.

A LOOK AHEAD

H.pylori infection as a model to study the impact of infections on anti-tumor immunological mechanisms

In this study, we have shown that the ABH and Lewis antigenic system polymorfism of the host is associated with the *H.pylori* and CagA seroprevalence, and with the ability of the host to respond to *H.pylori*. The expression of cancer-associated T glycotope in *H.pylori* further suggests that blood-group antigens and their derivatives are involved in *H.pylori*-host interplay with the relevance to clinical outcome of the infection. Moreover, the Lewis phenotype-dependent enhancement of natural IgG mmune response to T antigen in *H.pylori*-seropositive individuals was found to be beneficial for patients at early stages of gastric cancer. We reported recently that natural immune response to another cancer-related alpha-galactosyl epitope (Galα1,3-Gal) is also up-regulated in *H.pylori*-infected individuals (Kurtenkov et al., 2001). Thus it appears that T antigen is not a unique example in this respect and may reflect a particular case in some common pathway.

In addition to ABH and Le antigens system, other host genotypic/phenotypic traits have been shown to be related to *H.pylori*-host relationships and clinical outcome of the infection: IL1 beta polymorphism (El Omar, 2001; Figueiredo et al., 2002) TNF-alpha promoter polymorphism (Yea et al., 2001). These results provide indirect evidence of the increased pro-inflammatory effect associated with some allels and suggest that host genotyping may provide an important tool in defining disease risk. It is evident that all above mentioned facts are closely related to the immune mechanisms both humoral and cell-mediated.

In addition to the expression of tumor-associated antigens in *H.pylori*, another hypothetical mechanism may be considered for an enhancement of tumor-related immune response associated with *H.pylori* infection. Theoretically, the *H.pylori* glycosidases and/or glycosyltransferases may create the tumor-related epitopes on gastric glycoconjugates. It has been shown that *H.pylori* strains differ in their fucosidase activity (Dwarakanath et al., 1995). Schematically, the hypothetical sequence of events could be as follows: the alteration of gastric glycoconjugates glycosylation due to *H.pylori* glycosidases and/or transferases — the expression of autoimmunogenic tumor-related carbohydrate and/or peptide epitopes — the induction of autoimmune response to these epitopes. Another possibility is the modulation of *H.pylori* or host glycosidases expression and function in inflammatory conditions possibly via the formation of isoforms, or glycoforms.

Degradation of gastric mucins and alteration of their glycosylation may lead to the expression of autoimmunogenic peptide epitopes such as MUC1. Humoral immune response to MUC1 has been shown to increase the survival rate of patients with early breast cancer (von Mensdorff-Pouilly et al., 2000b).

One can not exclude that such a mechanism may operate in *H.pylori*-infected mucosa. Since the beneficial effect of *H.pylori* infection on the survival of surgically treated patients with early gastric cancer is associated with humoral immune response to tumor-related T epitope, it should be expected that this systemic impact of *H.pylori* might also operate in patients with other malignancies. It is reasonable to look for such associations to further confirm the systemic impact of *H.pylori* infection on natural immune defense against cancer.

Several studies are now in progress to evaluate this hypothesis, to provide better evidence for the idea that host-microbial interactions may modulate tumor-related immune mechanisms, to define better disease risk and to target *H.pylori* eradication to high-risk individuals. In many respects, the *H.pylori* infection may be considered as a good clinical and experimental model for that.

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KOKKUVÕTE

Humoraalne immuunvastus *Helicobacter pylori*'le: peremehest ja mikroobist sõltuvate tegurite uuring

Helicobacter pylori avastamine 20 aastat tagasi (1983.a.) muutis tugevasti ettekujutust mao ja kaksteistsõrmiksoole krooniliste haiguste patogeneesist. Sellesse infektsiooni on nakatunud üle 50% meie planeedi elanikest, Eestis ligikaudu 80% täiskasvanud elanikkonnast. *H.pylori* infektsiooni seos gastroduodenaalse patoloogiaga ei tekita enam kahtlusi. Põletik ja immunoloogilised mehhanismid on keskseks lüliks patogeneesiahelas põhjustades mao limaskesta rakkude kahjustusi, mis viivad atroofia, autoimmuunsete protsesside, düsplaasia ja maovähi tekkimiseni. Vähe on teada patogeneetilistest mehhanismidest, mis määravad infektsiooni kliinilise tulemuse individuaalsel tasemel. Probleem on tähtis ka praktilisest küljest vaadatuna kuna selle lahendusest sõltub, keda ja millal *H.pylori* infektsioonist ravida ning kas üldse ravida. Infektsiooni kliinilise tagajärje erinevuste selgitamiseks on vähemal kolm võimalust: (1) *H.pylori* muutlikkus ja virulentsemate bakteritüvede olemasolu või eelistatult teatud haiguse patogeneesis osalevad bakteritüved; (2) peremeesorganismi unikaalne või muutunud reaktsioon nakkusele, mis viib teatud kliinilise tagajärjeni; (3) teised tegurid nagu keskkonna mõjurid, mis võivad moduleerida bakteri ja peremeesorganismi vahelisi suhteid. Siiski näib, et erinevate omaduste teatud kombinatsioon määrab selle nakkuse kliinilise tagajärje.

Nii peremeesorganismi kui ka *H.pylori* suur geneetiline ja fenotüübiline polümorfism lubab *a priori* oletada erinevusi infektsiooni kliinilises tagajärjes. Viimase paari aasta jooksul on saanud selgeks, et "virulentsustegurite mudel" üksi ei suuda seletada *H.pylori* infektsiooni kliiniliste tagajärgede erinevusi indiviidide vahel. Sellest räägib ka seose puudumine *H.pylori* tuntud virulentsustegurite (nende geenide ja alleelide) olemasolu ning konkreetse maohaiguse vahel aga samuti ka segainfektsioonide (erineva genotüübiga *H.pylori* tüvede eksisteerimine maos) suur sagedus. El Omar (2001) ja Figueiredo et al. (2002) näitasid, et inimese geneetiline polümorfism (IL-1B geen, TNF-α promootori polümorfism) on seotud eelsoodumusega teatud *H.pylori* põhjustatud haigustele. *H.pylori* infektsiooni pikaajaline püsimine viib maksimaalse vastastikuse adapteerumiseni, mis suurendab veelgi selle vahekorra unikaalsust.

H.pylori vastu suunatud immuunvastus peegeldab nii *H.pylori* antigeenset spektrit kui ka peremeesorganismi võimet vastata sellele tüüpilisele "aeglasele" infektsioonile, kus peremeesorganism on peamiseks teguriks (Nguen et al., 1999; Blaser 2000, 2002). Meie uurimistöös olid erilise tähelepanu all peremeesorganismi omadused, mis võivad mõjutada bakter-peremees omavahelist vahekorda. Uurisime ABH ja Lewis veregrupi antigeenidega seotud süsivesikantigeene ning nende derivaate, mis on ekspresseeritud nii *H.pylori* pinnal kui ka peremehe mao mutsiinidel ja mukoosa rakkude glükokonjugaatidel. Mut-

siinid on *H.pylori* normaalseks *in vivo* elukeskkonnaks ning kahjustuvad kõige rohkem erinevate maohaiguste korral. See on väga polümorfne süsteem, mida kontrollivad ABH, Le ja Se/se geenid ning mis on kaasatud *H.pylori* adhesiooni ja kolonisatsiooni läbi BabA ja SabA adhesiinide. Need süsivesikantigeenid võivad olla sihtmärgiks autoimmuunsetele protsessidele, mis omakorda on tihedalt seotud mao mukoosa kahjustustega. Loomulikku ja omandatud immuunvastust autoimmunogeensetele süsivesikepitoopidele võib vaadelda kui integraalset näitajat, mis peegeldab nende epitoopide ekspressiooni *H.pylori*'l ning peremeesorganismi võimet neid ära tunda ja vastata. Selles mõttes on immuunvastus heaks test-süsteemiks peremehe polümorfismi mõju hindamisel peremees-mikroob vahekorrale.

Eesmärgid

Meie töö eesmärgiks oli uurida humoraalset immuunvastust *H.pylori* antigeenidele gastroduodenaalsete haigustega patsientidel ja veredoonoritel erilise tähelepanuga peremehe veregrupiga seotud antigeenide polümorfismile, mis võivad olla tegevad *H.pyloriga* seotud haiguste patogeneesis ja kliiniliste tagajärgede tekkimisel.

Töö põhilised ülesanded:

1. Hinnata *H.pylori* infektsiooni ja selle virulentsemate CagA positiivsete tüvede levikut gastroduodenaalsete haigustega patsientide (maovähk, peptiline haavand, krooniline gastriit) ja veredoonorite seas arvestades isiku ABH ja Lewis veregrupi fenotüüpi ning Se/se sekretoorset staatust.
2. Uurida *H.pylori* infektsiooni ja peremehe Le(a,b) fenotüübi mõju Lewis tüüp 2 antigeenidele suunatud immuunvastusele.
3. Uurida *H.pylori* infektsiooni võimalikku süsteemset toimet kasvajaseoselisele veregrupiga seotud Thomsen-Friedenreichi antigeenile (Galβ1,3GalNAc-O-Ser/Thr) suunatud loomulikule immuunvastusele erinevates kliinilistes gruppides.
4. Kontrollida hüpoteesi, et *H.pylori* võib enda pinnal ekspresseerida kasvajaseoselist Thomsen-Friedenreichi antigeeni.
5. Hinnata maovähihaigete elulemust arvestades *H.pylori* seroloogilist staatust ja Thomsen-Friedenreichi antigeenile suunatud loomulike antikehade taset.

Materjal ja meetodid

Uuriti 204 patsienti mao healoomuliste haigustega, 281 maovähihaiget ning 306 juhuslikult valitud veredoonorit. Kasvaja staadiumi määramine ja morfoloogiline iseloomustus põhinesid histopatoloogilisel pTNM klassifikatsioonil. Peptilise haavandi diagnoos põhines endoskoopilisel uuringul. Kroonilise gastriidi

haigetel hinnati mao korpusest ja antrumist võetud proove histoloogiliselt vastavalt Sydney süsteemile. *H.pylori* standardse tüve NCTC 11637 ning samuti *H.pylori* kliinilised isolaadid kultiveeriti mikroaerofiilsetes tingimustes. Antigeenina kasutati *H.pylori* membraani antigeene (glütsiini ekstrakt), CagA rekombinantset fragmenti ning sünteetilisi T ja Lewis a,b,x,y hapteenide konjugaate polüakrüülamiidiga (PAA). Anti-*H.pylori* IgG antikehade tset määrati imunoensüümmeetodil (ELISA). Antikehade spektrit uuriti SDS elektroforeesi ja immunoblottingu meetodil. ABH ja Lewis fenotüüp määrati erütrotsüütidel monokloonsete antikehadega. Sekretoorne staatus määrati ELISA meetodil H antigeeni sisalduse järgi süljes. Lewis tüüp 2 ning Thomsen-Friedenreichi süsivesikantigeenidele suunatud IgG ja IgM antikehade taset hinnati ELISA meetodil hapteen-PAA konjugaatidega. T-antigeeni ekspressiooni H.pylori pinnal määrati immunoblottingu meetodil terve rea mono- ja polükloonsete antikehadega ning samuti cell-ELISA meetodil. Saadud andmeid töödeldi parameetriliste ja mitteparameetriliste statistiliste meetoditega. Vähihaigete elulemust hinnati Kaplan-Meyeri meetodil (log-rank test).

Tulemusi analüüsiti kahte moodi: (1) võrdlesime tulemusi *H.pylori* seronegatiivsete ja -positiivsete isikute vahel igas uuringugrupis (doonorid, patsiendid), et leida erinevusi, mis on seotud *H.pylori* infektsiooniga (2) võrdlesime tulemusi uuringugruppides nakatunud ja nakatumata isikute hulgas, et leida haiguse-spetsiifilisi muutusi. Peremehest sõltuvaid tegureid, ABO(H) ja Lewis (a,b) fenotüüpi, sekretoorset staatust, T-antigeen spetsiifiliste IgG ja IgM ning Lewis tüüp 2 antikehade taset, kasvaja morfoloogiat, haiguse staadiumi ning vähihaigete elulemust võrreldi *H.pylori* seroloogilise staatuse, anti-*H.pylori* antikehade taseme, CagA staatuse, immunoblottingu tulemuste ja T-antigeeni ekspressiooniga *H.pylori* pinnal.

Peamised tulemused

Põhja-Eestis leiti seedetrakti haigustega ja sama vanusegrupi veredoonorite seas 75.5–86.2% *H.pylori* seropositiivseid isikuid, mis ei olnud aga seotud mingi kindla haigusega. Maovähihaigete hulgas langes *H.pylori* seropositiivsus sõltuvalt kasvaja arengu staadiumist. Immunoblottingu tulemused näitasid virulentsema CagA positiivse tüve suuremat esinemissagedust (74.2–91.2%) gastroduodenaalse patoloogiaga v.a. atroofilise gastriidiga (56.1%) patsientidel ning kaugelearenenud maovähiga haigetel (62.5%). Kõige rohkem CagA seropositiivseid isikuid oli mao- (91.2%) ja kaksteistsõrmiksoole (81.3%) haavandiga patsientide seas. Vähihaigetel vähenes risti-reageerivate (33–66kDa) antigeenide tase (määrati immunoblottingu meetodil).

H.pylori NCTC 11637 membraani antigeenidele suunatud immuunvastust mõjutasid tugevasti peremehe ABH ja Lewis(a,b) fenotüübid. Mõju oli pigem üldine kui haiguse spetsiifiline. Lewis (a+b-/mittesekreetor) fenotüübiga doonorite seas oli märgatavalt rohkem *H.pylori*-seronegatiivseid isikuid ning

madalam anti-*H.pylori* IgG immuunvastus kui Le(a-b+)/ sekreetor fenotüübiga isikutel. Need andmed kinnitavad kaudselt *H.pylori* Le(b) adhesiini BabA osalust mao koloniseerimisel *H.pylori*'ga. Teisest küljest näitas see, et peremehe tugev immuunvastus ei ole otseselt seotud *H.pylori* resistentsusega.

Immunoblottingu ja immunoensüümmeetodil (CagA rekombinantse fragmendiga) saadud andmetel oli A veregrupiga doonorite ja mao healoomuliste haigustega patsientide seas oluliselt vähem CagA seropositiivseid isikuid võrreldes teiste ABO(H) fenotüüpidega doonoritega. Maovähihaigete seas oli see seos vähem väljendunud ning anti-CagA immuunvastus vähenes haiguse staadiumi arenedes olenemata vanusest, soost või kasvaja histoloogilisest iseloomustusest. Nende erinevuste aluseks olevad mehhanismid nõuavad edasisi uuringuid.

Le(b-) fenotüübiga veredoonorite seas oli oluliselt kõrgem CagA seropositiivsete isikute protsent võrreldes sama uurimisrühma Le(b+) fenotüübiga isikutega. Seega on Le(b-) fenotüübiga isikutel eelsoodumus nakatuda CagA positiivse *H.pylori* tüvega.

Eelmainitud Le(a+b-) fenotüübiga isikute suhteline resistentsus selle infektsiooni suhtes ei ole nende andmetega vastuolus kuna on loogiline, et *H.pylori* infektsiooni suhtes resistentsemad isikud võivad nakatuda ainult virulentsemate tüvedega. Krooniliste maohaigustega patsientide sekretoorne (Se/se) staatus ei olnud seotud CagA seroloogilise staatusega kuigi kaksteistsõrmiksoole haavandiga patsientide seas oli oluliselt rohkem mitte-sekreetoreid.

Lewis 2 tüüpi determinantidele [Le(x), Le(y)] suunatud IgG immuunvastus oli seotud nii peremeesorganismi *H.pylori* seroloogilise staatuse kui ka Lewis(a,b) fenotüübiga. Le(b+)/sekreetor fenotüübiga isikutel oli tugevam Le(x) ja Le(y) epitoopidele suunatud immuunvastus olenemata *H.pylori* seroloogilisest staatusest. *H.pylori* nakatunud Le(b-) fenotüübiga isikute seas oli rohkem tugevaid reageerijaid Le(x) antigeenile võrreldes sama uurimisrühma *H.pylori* nakatumata isikutega. Võib oletada, et nendel isikutel on Le(x) epitoop kõige tõenäolisemaks sihtmärgiks *H.pylori* infektsiooniga seotud autoimmuunsetele reaktsioonidele.

H.pylori infektsioon on seotud tugevama immuunvastusega kasvajaseoselisele Thomsen-Friedenreichi (T) antigeenile (Galβ1,3-GalNAcα/β-O-Ser/Thr). Selline süsteemne toime sõltub permehe Lewis(a,b) fenotüübist ning on osaliselt haiguse-spetsiifiline. Anti T antikehade tase oli kõrge kõikides *H.pylori*-seropositiivsetes uurimisrühmades. Kõige madalam antikehade tase oli peptilise haavandiga haigetel olenemata *H.pylori* seroloogilisest staatusest. Suuremad erinevused *H.pylori* seropositiivsete ja -negatiivsete doonorite võrdlemisel leiti Le(b-) fenotüübiga isikute seas: anti-T spetsiifiliste IgG antikehade tase oli kõrgem nakatunutel. *H.pylori* infektsiooni ravi tulemusena antikehade tase langes osadel peptilise haavandi haigetel. Saadud andmed näitavad, et *H.pylori* infektsioon võib moduleerida loomulikke kasvajavastaseid immunoloogilisi mehhanisme.

Tõendasime immunoloogiliselt kasvajaseoselise autoimmunogeense T-epitoobi ekspressiooni *H.pylori* membraani glükokonjugaatidel ja selle ekspressiooni seotust loomuliku anti-T immuunvastuse muutustega nakatunud isikutel. T-spetsiifiliste monokloonsete antikehadega andsid värvusreaktsiooni peamiselt kaks proteiini 68 kDa ja 58 kDa. Erinevad *H.pylori* tüved ekspresseerivad T-epitoopi erineval määral, mis osaliselt sõltub maohaigusest: 68kDa proteiini suurem ekspressioon oli seotud raskema patoloogiaga (kaksteistsõrmiksoole haavand, maovähk).

Maovähi varases staadiumis patsientide elulemus (kuni 6 aasta jälgimise andmetel) oli oluliselt parem *H.pylori* seropositiivsete patsientidel. See fenomen oli seotud T-antigeen spetsiifilise IgG immuunvastuse tugevnemisega *H.pylori* nakatunud isikutel. Nii võib öelda, et *H.pylori* infektsioon on kaudselt seotud mao kantserogeneesiga moduleerides loomulikke kasvajavastaseid immuunmehhanisme ning selline toime võib olla kasulik maovähihaigetele.

Kõik ülaltoodu lubab teha kaks üldist järeldust:

(1) peremeesorganismi veregrupi antigeenide (ABH ja Lewis) polümorfism mõjutab tugevalt peremehe immuunvastust *H.pylori* antigeenidele, infektsiooni esinemissagedust ning inimese eelsoodumust nakatuda teatud genotüübiga H.pyloriga (näiteks CagA+ tüvedega nakatuvad sagedamini Le(b-) fenotüübiga isikud ja harvemini A veregrupiga isikud). Peremeesorganismi ABH ja Lewis fenotüüpide moduleerivat efekti tuleks arvestada võrdlevatel s.h. ka epidemioloogilistel *H.pylori* infektsiooniga seotud haiguste uuringutel.

(2) Kasvajaseoselise Thomsen-Friedenreichi antigeeni abil näidati, et *H.pylori* infektsioon võib stimuleerida kasvajavastaseid immuunmehhanisme ning parandada maovähihaigete elulemuse näitajaid. Sellist süsteemset efekti võib oodata ka vähi mitte-mao lokalisatsioonide korral. Arvame, et *H.pylori* infektsioon on mugavaks mudeliks infektsiooni mõju uurimisel loomulikele kasvajavastastele immuunmehanismidele.

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PUBLICATIONS

I

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IgG IMMUNE RESPONSE TO *HELICOBACTER PYLORI* ANTIGENS IN PATIENTS WITH GASTRIC CANCER AS DEFINED BY ELISA AND IMMUNOBLOTTING

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Helicobacter pylori infection is considered to be a risk factor for gastric cancer. A high prevalence of _H. pylori_ infection and high gastric-cancer incidence are characteristic of the Estonian population. To evaluate the relationship between these 2 events, we studied the seroprevalence of _H. pylori_ in gastric cancer patients (n = 182) and in healthy blood donors (n = 306). A relative anti-_H. pylori_ IgG antibody activity, as detected by ELISA and immunoblot patterns, was correlated with age, stage of the disease and tumor morphology. A significantly higher _H. pylori_ seroprevalence was found in patients in the early stages of tumor development compared with both advanced cancer patients and controls. No significant difference in _H. pylori_ seroprevalence between patients with the intestinal and diffuse types of tumor growth was observed. A decline in the recognition of putatively cross-reacting (33–66 kDa) antigens was noted in the cancer group. The response to vacuolating toxin-related 85-kDa and CagA 120-kDa protein antigens was not altered and was observed more often in the younger group of cancer patients.
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H. pylori gastric infection is supposed to be a risk factor for gastric cancer (Correa *et al.*, 1990; Eurogast Study Group, 1993; Parsonnet, 1993; IARC, 1994). The most convincing data for this supposition reside in the concordant results of prospective cohort studies in different high- and low-risk populations which had been followed over 6–14 years for later development of gastric cancer (IARC, 1994). A significantly higher risk of gastric cancer development (odds ratio 2.8–6) was found for *H. pylori*-infected individuals compared with *H. pylori*-negative controls, adjusted by age, sex and date of blood sampling.

A high prevalence of *H. pylori* infection and a high incidence of gastric cancer are both characteristic of the Estonian population (Maaroos *et al.*, 1990; Rahu and Aareleid, 1990; Vorobjova *et al.*, 1994). The relationship between these 2 events is unclear. We studied *H. pylori* seroprevalence in gastric cancer patients and controls by ELISA and further analyzed anti-*H. pylori* IgG reactivity patterns by immunoblotting, using a pool of acid glycine *H. pylori* extracts which contained the major surface-exposed immunoreactive *H. pylori* antigens (Lelwala-Guruge *et al.*, 1992). The data were correlated with age, stage of the disease and tumor morphology.

MATERIAL AND METHODS

Subjects

Serum samples from 182 consecutive patients (105 men, 77 women; median age 62; range 21–87 years) with histologically verified non-cardiac gastric cancer diagnosed in the Estonian Cancer Center and from 306 healthy blood donors and healthy employees (132 men, 174 women; median age 51; range 18–72 years) were examined. Donors were not asked about the presence of abdominal complaints. The patients and controls were divided into 5 10-year age groups (see Table 1). Gastric carcinoma was classified histologically into the intestinal (n = 102) and diffuse (n = 80) types, according to the system of Lauren (1965). Tumor staging was based on the histopathological (pTNM) classification system (Hermanek and Sobin, 1987).

Serum specimens

Sera were collected from all subjects by centrifugation of clotted venous blood after 2 hr incubation at 37°C and stored at −20°C.

Antigen preparation

A glycine cell-surface antigen extraction of *H. pylori* strain NCTC 11637 was performed according to Logan and Trust (1983). The strain was grown on GAB-CAMP reference agar at 37°C for 2 days in plastic jars filled with a gas mixture of 5% O_2, 10% CO_2 and 85% N_2. The antigen was a pool of 9 different preparations.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were examined by ELISA as described earlier (Lelwala-Guruge *et al.*, 1990). In brief, the 96-well flat-bottomed microtiter plates (Maxi Sorp, Nunc, Roskilde, Denmark) were coated with 0.1 ml of antigen (5 μg/ml) per well and incubated at 4°C overnight. Alkaline phosphatase conjugated anti-human IgG (Dako, Glostrup, Denmark) and p-nitro-phenyl-phosphate (Sigma, St. Louis, MO) substrate were used. A pool of human IgG (Kabi, Stockholm, Sweden) was used in each ELISA plate as a positive control for 100 units. Relative antibody activity (RAA) values above 25 were regarded as positive.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed in 12% polyacrylamide separating gel according to Laemmli (1970). A gel was loaded with *H. pylori* glycine extract previously mixed with 0.07 M TRIS HCl (pH 6.8) containing glycerol, SDS, bromphenol blue and mercaptoethanol, heated for 3 min at 90°C and cooled. Protein molecular weight standards (Promega, Madison, WI) were diluted in the same sample buffer without mercaptoethanol and treated similarly. After separation, the proteins were blotted onto a nitrocellulose sheet, 0.45 μm (Schleicher and Schuell, Dassel, Germany) in a semi-dry electroblotter (Millipore, Sundbyberg, Sweden). The nitrocellulose membrane was saturated as described by Rucheton *et al.* (1992) and, after drying, cut into strips. The strips were washed and incubated with patients' sera (1:76), overnight at 4°C under agitation. In each plate one strip was incubated with standard IgG (Kabi). The strips were washed and incubated for 2 hr at 4°C with peroxidase-labelled rabbit anti-human IgG (Dako) and developed with carbazol (Merck, Darmstadt, Germany).

Statistical analysis

Statistical comparisons between the groups were performed by the Chi-square test. The differences were considered to be significant when $p < 0.05$.

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TABLE I – PREVALENCE OF *H. PYLORI* IgG SERUM ANTIBODIES IN 182 GASTRIC CANCER PATIENTS AND 306 CONTROLS BY AGE AND DISEASE STAGE

Group	Age (years)											
	< 40		40–49		50–59		60–69		> 69		All	
	HP+[1]	*HP−*[1]	*HP+*	*HP−*	*HP+*	*HP−*	*HP+*	*HP−*	*HP+*	*HP−*	*HP+*	*HP−*
Gastric cancer n = 182	10 (100.0)[3]	0	12 (80.0)	3	36 (81.8)	8	59 (88.1)	8	36 (78.3)	10	153 (84.1)	29
Stage I[2] n = 26	3 (100.0)	0	4 (100.0)	0	4 (100.0)	0	7 (87.5)	1	7 (77.8)	2	25 (89.3)	3
Stage II n = 40	2 (100.0)	0	0	0	12 (100.0)	0	11 (91.7)	1	13 (92.85)	1	38 (95.0)	2
Stage III n = 35	0	0	1 (50.0)	1	3 (50.0)	3	18 (90.0)	2	6 (85.7)	1	28 (80.0)	7
Stage IV n = 79	5 (100.0)	0	7 (77.8)	2	17 (77.3)	5	23 (85.2)	4	10 (62.5)	6	62 (78.5)	17
Controls n = 306	41 (77.4)	12	105 (80.2)	26	86 (79.63)	22	11 (84.6)	2	0	1	243 (79.4)	63

[1]*H. pylori*-seropositive/seronegative individuals. For *H. pylori* seropositivity criteria, see text.–[2]Tumors were classified according to TNM criteria for carcinoma of the stomach, established by the International Union Against Cancer (Hermanek and Sobin, 1987).–[3]In parentheses: per cent of the *H. pylori*-seropositive individuals.–The difference in *H. pylori* seroprevalence: stage I + II *versus* stage III + IV, $\chi^2 = 4.99$ df = 1, $p = 0.025$; stage I + II *versus* controls, $\chi^2 = 5.69$, df = 1, $p = 0.017$, as calculated by Chi-square test.

RESULTS

The overall *H. pylori* seropositivity rates were 79.4% and 84.1% for controls and gastric-cancer patients, respectively, and did not differ significantly ($p = 0.25$). However, the *H. pylori* seroprevalence in the patients in early stages of cancer (I + II) was significantly higher (63/68, 92.6%) than in either the patients in later stages of the disease (90/114 (78.9%); $\chi^2 = 4.99$, $p = 0.025$; odds ratio (OR) = 3.36, 95% CI (1.1–10.6) or controls (243/306 (79.4%), $\chi^2 = 5.69$, $p = 0.017$; OR = 3.26, 95% CI (1.2–9.4) (Table I). No significant age-related differences in the *H. pylori* seropositivity rate were found in controls and within the group of cancer patients at different stages of the disease. The youngest group of patients (< 40 yr) was small (n = 10, 5 of them in stage I–II) and all of them were seropositive. No differences between cancer patients and controls were found in any age-adjusted groups when the data were compared, irrespective of the disease stage. Thus, only the stage of the disease showed an association with *H. pylori* seroprevalence in patients with gastric cancer aged over 40. The total *H. pylori* seropositivity rate was similar for patients with the diffuse (83.7%) and the intestinal (84.3%) types of tumor growth according to Lauren's classification; furthermore, a similar decline in *H. pylori* seroprevalence in advanced cancer was revealed in both groups (Table II).

On immunoblotting, the protein profile consisted predominantly of 9 major bands with molecular weights of ~120, 85, 66, 62, 59, 47, 43, 33 and 28 kDa (Fig. 1). The significant differences between *H. pylori*-seropositive and -seronegative groups were mostly associated with bands of 33, 59 and 66 kDa for controls and with bands of 85 and 59 kDa for gastric-cancer patients (Table III). Three of 31 (9.7%) controls and 14 of 96 (14.6%) cancer patients did not recognize any bands at all. All of them, with the exception of 2 cancer patients, were *H. pylori* seronegative in ELISA.

The differences in *H. pylori* antibody spectra between gastric cancer patients and controls became visible if *H. pylori*-positive persons in both groups were compared (Table III). In the *H. pylori*-seropositive group, 100% of controls recognized the 33-kDa and 59-kDa bands, while in the cancer group the percentage was 61% and 73% ($\chi^2 = 10.8$, $p < 0.001$ and $\chi^2 = 6.0$, $p < 0.02$, respectively). The occurrence of low-molecular-weight bands (< 28kDa) was also significantly higher in *H. pylori*-seropositive control individuals ($\chi^2 = 6.6$, $p = 0.01$). The differences concern the 62–66 kDa region as well. The number of persons recognizing these bands was also significantly higher in the control group ($p < 0.05$). A decreased recognition of 28–66 kDa region bands was more pronounced in *H. pylori*-seropositive patients at stage III–IV compared with the seropositive controls, especially for 33-kDa and 66-kDa antigens: cancer group, 70.7% and 46.3%; controls, 100% and 75.9%; $p = 0.01$ and $p = 0.06$, respectively). No remarkable differences between *H. pylori*-negative patients and controls were found.

TABLE II – PREVALENCE OF *H. PYLORI* IgG SERUM ANTIBODIES IN PATIENTS WITH GASTRIC CANCER BY DISEASE STAGE AND HISTOLOGICAL TYPE OF TUMOR

Gastric cancer patients	Histological type of tumor			
	Diffuse type (n = 80)		Intestinal type (n = 102)	
	HP+[2]	*HP−*[2]	*HP+*	*HP−*
Stage I n = 28	15 (93.8)[3]	1	10 (83.3)	2
Stage II n = 40	12 (92.3)	1	26 (92.3)	1
Stage III n = 35	6 (75.0)	2	22 (81.5)	5
Stage IV n = 79	34 (79.1)	9	28 (77.8)	8
All n = 182	67 (83.7)	13	86 (84.3)	16

[1]According to Lauren classification (1965).–[2]*H. pylori*-seropositive/seronegative individuals. For *H. pylori* seropositivity criteria, see text.–[3]In parentheses: percent of *H. pylori*-seropositive individuals.

The vacuolating cytotoxin-associated 120-kDa protein band was recognized by 55% of the *H. pylori*-seropositive gastric cancer patients and by 69% of the controls, but 8 of 24 (33%) *H. pylori*-negative cancer patients and 4 of 8 (50%) *H. pylori*-negative healthy controls recognized this band as well. A higher frequency of the 85-kDa band in blots of sera from cancer patients under 50 years of age (13/15, 86.7%) was noted compared with age-related controls (7/14, 50%, $p = 0.08$). This band was recognized only by 53.1% (43/81) of the cancer patients above this age ($\chi^2 = 4.57$, $p = 0.032$, compared with the younger group of patients). A similar age-related trend was noted for 120-kDa protein: 73.3% of patients aged below 50 yr and 56.2% of those above this limit show this band on blotting.

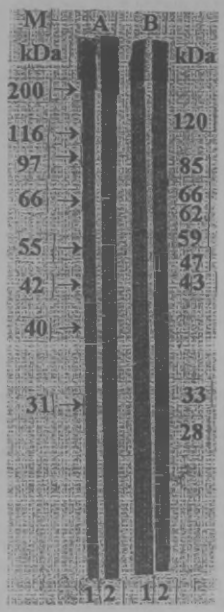

FIGURE 1 – *H. pylori* immunoblots incubated with sera from gastric cancer patients (1) and healthy persons (2). M, marker proteins; A, *H. pylori*-seronegative persons; B, *H. pylori*-seropositive persons. Molecular masses of M, standards (left) and the major *H. pylori* antigens (right).

but the opposite result was obtained in age-subgroups of controls (57.1% and 70.1%, respectively). No other age-related differences were found in the patients and controls. In contrast to the 28–66 kDa area, the frequency of 85-kDa and 120-kDa protein bands was similar for *H. pylori*-seropositive patients at the early and late stages of gastric cancer (in the range of 62–70%).

As in the case of ELISA, we found no difference in blotting patterns between the patients according to tumor histology. *H. pylori*-seropositive cancer patients with intestinal-type tumors showed a higher percentage of recognition of the 120-kD but not the 85-kD protein band as compared with the group of patients with diffuse-type cancers (72% *vs.* 59%), but the difference was not significant.

DISCUSSION

The data show that the *H. pylori* seropositivity rate in patients with gastric cancer, as defined by ELISA, is related to the stage of the disease and is significantly higher at early stages of tumor development, though the overall *H. pylori* seropositivity in the cancer group did not differ significantly from that seen in controls. No age-related differences were found in controls: the *H. pylori* seroprevalence almost reached its maximum in controls under 40 years of age, remained at a level of 79–84% in those aged up to 70 years and did not differ significantly from that seen in the age-related groups of cancer patients. The same was true for the differences between 10-year age subgroups of patients in any stage of cancer except the youngest group of patients (under 40 yr) who were all seropositive irrespective of the disease stage. Thus, it appears that the stage distribution is a very important factor influencing the *H. pylori* seroprevalence in gastric-cancer patients over 40 years of age.

A higher rate of histologically detected *H. pylori* positivity was reported for early than for advanced gastric cancer (Caruso and Fucci, 1990; Sakaki *et al.*, 1993). Asaka *et al.* (1994) also found that the *H. pylori*–gastric cancer association is restricted to early gastric cancer. It is noteworthy that such an association with the highest odds ratio was observed in subjects under 50 years of age, though the decrease in odds ratio with age was mainly due to an increase in the seropositivity rate in controls (Kikuchi *et al.*, 1995). Our data are in accordance with these findings. It is likely that, in some gastric cancer patients, the infection may have disappeared by the time the cancer is diagnosed, leading to a classification of "false seronegatives," especially in the group of older patients and/or at the later stages of cancer. In spite of all these findings, the distribution of gastric-cancer patients by stage is still often overlooked in many case-control studies. It could be expected that the situation at the early stages of neoplasia reflects the *H. pylori*–host relationships in a more realistic way because many secondary events associated with advanced cancer are excluded (an altered microenvironment due to aberrant glycosylation, colonization of the stomach by bacteria other than *H. pylori*, microbial overgrowth, tumor-induced immunosuppression). Otherwise, an underestimation of *H. pylori* seroprevalence in cancer groups may occur.

It should be mentioned that *H. pylori* seroprevalence in our controls was similar to that obtained in the population-based studies performed recently in Estonia (Maaroos *et al.*, 1990; Vorobjova *et al.*, 1994). Keeping in mind that the controls used in this study might include some individuals with non-ulcerous dyspepsia or even with peptic ulcers, the *H. pylori* seroprevalence in this group is possibly overestimated. However, in spite of a very high *H. pylori* seroprevalence in controls, there was a significantly higher seropositivity rate in patients in early stages of gastric cancer.

We found no significant difference in *H. pylori* seroprevalence between diffuse- and intestinal-type gastric cancers. The prevalence of *H. pylori* infection in patients with intestinal-type adenocarcinomas has been observed by many investigators but not confirmed by others (IARC, 1994). We noted only a slight prevalence of recognition of 120-kDa antigen by serum of patients with intestinal-type tumor growth.

A higher proportion of <28, 33, 59 and 62–66 kDa bands was typical in sera of controls compared with those of cancer patients. These putatively cross-reacting antigens showed a close association with *H. pylori* seropositivity in ELISA. The cell-surface urease, flagellar and heat-shock proteins have similar molecular masses and were shown to have properties similar to those of some other species (Dunn *et al.*, 1989; Maeland *et al.*, 1993). By contrast, the frequency of the vacuolating-toxin-related 85-kDa band was even increased in patients with gastric cancer, especially in patients under 50 years of age. The same was true for 120-kDa protein. Hence, the IgG immune response to these cytotoxin-related *H. pylori* antigens remains stable in cancer patients, possibly due to a better immunogenicity of these antigens or their enhanced production in gastric-cancer patients.

In spite of considerable phenotypic conservation, at least 4 phenotypic characteristics may be different among *H. pylori* strains: the structure of lipopolysaccharide, expression of vacuolating cytotoxin and cagA-encoded protein (120–140 kD) and the ability to activate neutrophils (Blaser, 1994). The vacuolating cytotoxin-related 85–87 kDa and 120–130 kDa protein antigens are present in 50–70% of *H. pylori* strains (Tummuru *et al.*, 1993; IARC, 1994). The expression and systemic IgG or mucosal IgA recognition of the 120-kDa protein was shown to be associated with active gastritis, peptic

TABLE III - DISTRIBUTION OF THE MAJOR *H. PYLORI* ANTIGENS DETECTED BY IMMUNOBLOTTING WITH SERA OF *H. PYLORI*-SEROPOSITIVE (HP+) AND *H. PYLORI*-SERONEGATIVE (HP−) CANCER PATIENTS AND CONTROLS

Mol. mass (kDa)	Gastric cancer			Healthy blood donors		
	All n = 96	*HP*− n = 24 (25)	*HP*+ n = 72 (72)	All n = 31	*HP*− n = 8 (26)	*HP*+ n = 23 (74)
120	48 (50)[3]	8 (33)	40 (55)	20 (64)	4 (50)	16 (69)
85	56 (58)	7 (29)	49 (68)[1]	17 (54)	3 (37)	14 (61)
66	41 (42)	7 (29)	34 (47)[2]	19 (61)	2 (25)	17 (73)[1]
62	38 (39)	10 (41)	28 (38)[2]	17 (54)	2 (25)	15 (65)
59	64 (66)	11 (45)	53 (73)[1,2]	27 (87)	4 (50)	23 (100)[1,2]
47	60 (62)	11 (45)	49 (68)	23 (74)	5 (62)	18 (78)
43	28 (29)	4 (16)	24 (33)	10 (32)	1 (12)	9 (39)
33	57 (59)	13 (54)	44 (61)[2]	26 (83)	3 (37)	23 (100)[1]
28	59 (61)	11 (45)	48 (66)	18 (58)	3 (37)	15 (65)
<28	32 (33)	6 (25)	26 (36)[2]	18 (58)	2 (25)	16 (69)

[1]Significantly different from related *HP*− group as calculated by Chi-square test.–[2]Significantly different from *HP*+ group of blood donors.–[3]In parentheses, percent of seropositive cases within each subgroup.

ulceration and gastric cancer (Crabtree *et al.*, 1991, 1993). Some differences in anti-*H. pylori* IgG_1 and IgG_2 subclass antibody immune response were found between *H. pylori*-infected patients with duodenal ulcer and those without ulcer (Bontkes *et al.*, 1992). Immunoblot patterns of different *H. pylori*-positive sera, when tested with the same *H. pylori* extract, also showed appreciable differences (Dunn *et al.*, 1989). Taken together, these findings suggest that the antigenic differences between strains and/or host-dependent peculiarities in anti-*H. pylori* immune response may be differently implicated in the pathogenesis of various *H. pylori* infection-associated diseases.

However no direct evidence has been presented so far concerning the involvement of *H. pylori* toxins in tumor-inducing specific mechanisms or in damaging of the anti-tumor defense system. We have shown (Kurtenkov *et al.*, 1995) that *H. pylori* gastric infections are associated with a suppressed anti-Thomsen-Friedenreich antigen natural immune response, which is considered to be an important factor in the natural anti-tumor defense system (Springer, 1984). The sequence of events remains unclear: whether this suppression is induced by the *H. pylori* infection *per se*, or whether individuals with a low natural immune response to this tumor-associated antigen are more susceptible to *H. pylori* infection.

In conclusion, the distribution of cancer patients by stage is an important factor in the evaluation of anti-*H. pylori* systemic immune response of patients with gastric cancer and in comparisons between cancer patients and controls. Patients in the early stages of cancer should be examined in the first place to avoid an underestimation of *H. pylori* seroprevalence due to the possible modulation of the immune response in patients with advanced cancer. We suggest that "the early-stage—early-age situation" seems to be a more favorable condition for studying the *H. pylori* infection–gastric-cancer relationships. The cancer patients were characterized by a decline in recognition of putatively cross-reacting low-molecular-weight (<28, 33–66 kDa) *H. pylori* antigens. The systemic IgG immune response to toxin-related 85- and 120-kDa *H. pylori* proteins was not altered, and was observed more often in the younger group of cancer patients. These changes may be connected with the existence of *H. pylori* strains, preferably involved in gastric carcinogenesis, such as toxin-producing strains, or be related to the peculiarities of the host immune response. A further study of the cell-surface antigenic profile of *H. pylori* isolates derived from gastric-cancer patients and controls, as well as a comparison of the immune response to homologous and heterologous *H. pylori* antigens, are required to answer the question whether any cancer-specific signs of *H. pylori* gastric infection exist.

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II

Klaamas K, Kurtenkov O, Ellamaa M, Wadström T.
The *Helicobacter pylori* seroprevalence in blood donors related to Lewis (a,b) histo-blood group phenotype. Eur J Gastroenterol Hepatol 1997; 9: 367–370.

The *Helicobacter pylori* seroprevalence in blood donors related to Lewis (a,b) histo-blood group phenotype

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Objective: To study a possible association of the *Helicobacter pylori* seroprevalence with ABO(H) and Lewis (a,b) blood group phenotypes in blood donors.

Design: A cross-sectional study of blood donors using ABO(H) and Lewis (a,b) blood group phenotype as predictors.

Methods: ABO(H) and Lewis (a,b) blood group phenotyping was performed with monoclonal antibody. The *H. pylori* immunoglobulin G (IgG) antibody relative activity was evaluated by enzyme-linked immunosorbent assay (ELISA) using acid glycine extract from *H. pylori*.

Subjects: One hundred and fifty-nine randomly selected blood transfusion donors.

Results: The individuals with Lewis (a+b–)/non-secretor phenotype showed a significantly higher proportion of the *H. pylori*-seronegative subjects and a lower IgG immune response to *H. pylori* antigens as compared with the individuals of Lewis (a–b+)/secretor phenotype.

Conclusion: The Lewis (a,b) histo-blood group antigens are implicated in the mechanisms of naturally occurring resistance to *H. pylori* infection.

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Keywords: ABO and Lewis blood groups, secretor status, *H. pylori* infection

Introduction

Several *Helicobacter pylori* cell-surface proteins or adhesins have been considered to be involved in the colonization of gastric mucosa by *H. pylori*: sialic acid-specific lectin, adhesins to fucosylated blood group antigens, glycosaminoglycans and to the extracellular matrix proteins [1–3]. The appropriate host tissue receptors belong to the specific glycoconjugates in the mucin layer and on the gastric epithelial cells.

It is well known that individuals of blood group O and those who are non-secretors are over-represented among patients with peptic ulcers [4,5]. The secretor status is determined by the presence or absence of a fucose residue on the terminal galactose residues on the mucosal O-linked oligosaccharides and mucins in secretions, which in turn is determined by the inheritance of the appropriate α-1-2 fucosyl-transferase *Se* (secretor) gene [6,7]. At the phenotypical level the secretors belong to Lewis (Le) (a–b+) and those who are non-secretors to Le (a+b–) histo-blood-group phenotype. Individuals with Le (a–b–) phenotype may be secretors or non-secretors depending on the presence or absence of a *Se* gene [6].

The Le (b) histo-blood group antigen has been shown to mediate the *H. pylori* attachment to human gastric mucosa [1], suggesting that the Le (a+b–) /non-secretors may be more resistant to *H. pylori* infection than secretors. However, no convincing clinical support for this has been presented so far. In Estonia, more than 80% of adults over the age of 30 are *H. pylori* infected [8,9] and a rather low proportion of Le (a+b–)/non-secretors (~12%) is characteristic of the Estonian population [10]. We hypothesized that in populations with a very high prevalence *of H. pylori* infection, an over-representation of Le (a+b–)/non-secretors should be expected among the *H. pylori*-uninfected individuals mainly at the age when the *H. pylori* infection prevalence in a given population is close to its maximum. We report here that the Le (a+b–) /non-secretor histo-

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blood group phenotype is associated with a significantly lower proportion of *H. pylori*-infected blood transfusion donors as compared with Le (a–b+)/secretors, supporting the idea that the Lewis (a,b) blood group antigens may be involved in the natural resistance to *H. pylori* infection.

Material and methods

Blood samples were taken from 159 randomly selected blood transfusion donors (67 women and 92 men) ranging in age from 17 to 60 years, mean age 48 years. Donors were not asked about the presence of abdominal complaints. ABO(H) and Lewis blood group phenotype distribution is shown in Tables 1 and 2.

Sera were collected from all subjects by centrifugation of clotted venous blood after incubation for 2 h at +37°C and stored at –20°C.

ABO(H) and Lewis (a,b) phenotyping of erythrocytes was carried out using anti-A and anti-B monoclonal antibody (MonoCarb AB, Sweden) and anti-Lewis (a) and anti-Lewis (b) monoclonal antibody gel system (DiaMed, Switzerland) according to the instruction of the manufacturers.

A glycine cell surface antigen extraction of *H. pylori* strain NCTC 11637 was performed according to Logan and Trust [11]. The strain was grown on GAB-CAMP (Gonococcal agar base/*Campylobacter*) reference agar at 37°C for 2 days in plastic jars filled with a gas mixture of 5% oxygen, 10% carbon dioxide and 85% nitrogen. The antigen was a pool of nine different preparations.

Serum samples were examined by enzyme-linked immunosorbent assay (ELISA) as described earlier [12]. In brief, the 96-well flat-bottom microtitre plates (Maxi Sorp, Nunc, Roskilde, Denmark) were coated with 0.1 ml of antigen (5 μg/ml) per well and incubated at 4°C overnight. Alkaline phosphatase conjugated anti-human immunoglobulin G (IgG) (Dako A/S, Glostrup, Denmark) and *p*-nitro-phenyl-phosphate (Sigma, St Louis, MO) substrate were used. A pool of human IgG (Kabi AB, Stockholm, Sweden) was used in each ELISA plate as a positive control for 100 units. A relative antibody activity (RAA) value above 25 was regarded as positive. The individuals with so-called 'grey zone' RAA values (26–35) were also taken into account and together with the negative subjects were designated as 'low responders'.

Statistical comparisons between the groups were performed by the χ^2 test. Odds ratios (ORs) and their 95% confidence intervals (95% CI) were calculated to measure an association between the parameters studied. The differences were considered to be significant when $P<0.05$.

Results

The distribution of ABO(H) and Le (a,b) phenotypes among blood donors was similar to that obtained in a population-based study performed recently in Estonia [10]. A higher percentage of individuals with blood group A was found among the non-secretors (11 of 23, 47.8%) compared with both secretors (33 of 113, 29.2%) and the Le (a–b–) group (5 of 23, 21.8%), but the differences were not significant ($P>0.1$). The IgG immune response to *H. pylori* was not dependent on ABO(H) blood group phenotype if the data were analysed irrespective of Le (a,b) phenotype (Table 1). The proportion of low responders (RAA < 35) was also similar for individuals with O, A, B and AB blood group phenotypes: 15.0%, 23.0%, 21.7% and 23.5%, respectively ($P>0.05$).

The Le (a+b–)/non-secretor phenotype group contained a significantly higher proportion of the *H. pylori*-seronegative subjects than the Le (a–b+)/secretor group (Table 2). If the individuals with so-called 'grey zone' RAA values (RAA = 26–35) were included, the difference was even more pronounced (Fig. 1). Five of the six subjects in this group showed RAA values less than 30 (26, 26, 27, 29, 29) and four of them belonged to the Le (a+b–) subgroup. The individuals with Le (a–b–) phenotype reacted like Le (a–b+)/secretors showing the lowest proportion of *H. pylori*-seronegative results (8.7%). Thus it appears that Le (a+b–)/non-secretors are more resistant to *H. pylori* infection.

Interestingly, the proportion of low responders in a group of donors possessing Le (a) antigen was significantly higher than in Le (a)-negative individuals (43.5% and 15.4%, respectively; $\chi^2=8.14$, degrees of freedom (df) 1, $P=0.004$; OR = 4.2, 95% CI 1.5–12.1), whereas no significant difference was noted between Le (b)-positive and Le (b)-negative donors ($\chi^2=1.24$, $P=0.26$; OR = 0.6).

Table 1. The *H. pylori* serological status of 159 blood donors in relation to ABO(H) blood group phenotype.

ABO(H) blood type	*n*	*H. pylori* serological status: Negative	Positive*
O	67	10 (14.9)	57 (85.1)
A	52	9 (17.3)	43 (82.7)
B	23	4 (17.4)	19 (82.6)
AB	17	3 (17.6)	14 (82.4)
	159	26 (16.4)	133 (83.6)

*Relative antibody activity (RAA) >25. In parentheses: percentage of *H. pylori*-seronegative and -seropositive individuals in each ABO(H) subgroup.

Table 2. The *H. pylori* serological status of 159 blood donors depending on Le (a,b) blood group phenotype.

Le (a,b) phenotype	*n*	*H. pylori* serological status: Negative	Positive
Le (a+b–)	23	8 (34.8)*	15 (65.2)
Le (a–b+)	113	16 (14.2)	97 (85.8)
Le (a–b–)	23	2 (8.7)	21 (91.3)
	159	26 (16.4)	133 (83.6)

*Significantly different from the Le (a–b+) group of donors ($\chi^2 = 4.26$, df 1, $P = 0.038$, OR = 3.2, 95% CI 1.1–9.9). In parentheses: percentage of *H. pylori*-seronegative and -seropositive individuals in each Le (a,b) subgroup.

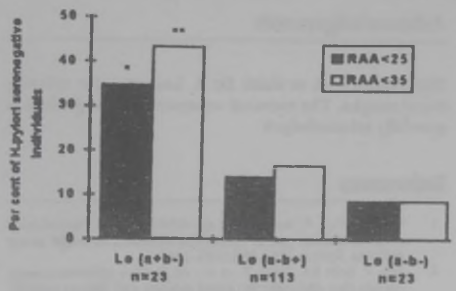

Fig. 1. The frequency of *H. pylori*-seronegative individuals among blood donors with different Le (a,b) blood group phenotypes. Dark bars, RAA < 25 as criterion for *H. pylori* seronegativity; light bars, RAA < 35 (low responders). *Significantly higher as compared with Le (a–b+)/secretors ($\chi^2 = 4.26$, df 1, $P = 0{,}039$; OR = 3.8, 95% CI 1.1–9.9). **Significantly higher as compared with both Le (a–b+)/secretors and Le (a-b–) group ($\chi^2 = 6.58$, and $\chi^2 = 5.52$, df 1, $P = 0.01$; OR = 3.8, 95% CI 1.3–11.0 and OR = 8.1, 95% CI 1.3–46.1, respectively).

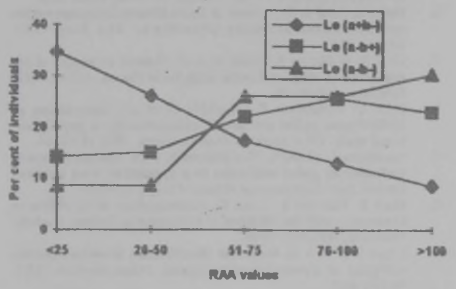

Fig. 2. Distribution of individuals with different Le (a,b) blood group phenotypes by relative *H. pylori* IgG antibody activity (RAA). The following groups of subjects were used for a cross-tabulation and χ^2 calculation: RAA < 25, 26–50, 51–75 and > 75. Subjects with RAA values equal to 76–100 and > 100 were combined (RAA > 75) because of the small number of donors with RAA values more than 100. Le (a+b–) vs. Le (a–b+): $\chi^2 = 9.33$, df 3, $P = 0.02$; Le (a+b–) vs. Le (a–b–): $\chi^2 = 8.91$, df 3, $P = 0.03$; Le (a–b–) vs. Le (a–b+): $\chi^2 = 1.45$, df 3, $P = 0.69$.

To exclude the influence of age on the *H. pylori* seropositivity rate we investigated mainly the older group of donors: 132 of 159 (83%) subjects tested were above 40 years of age. The difference in the proportion of *H. pylori*-seronegative individuals of different Le (a,b) blood group phenotypes was retained also in subgroups of individuals at 41–50 and 51–60 years of age: 40.0% and 33.3% for non-secretors; 16.7% and 13.3% for secretors, respectively. The percentage of the *H. pylori*-seronegative subjects was similar to that observed in the combined group (Fig. 1), thus indicating that this proportion was not appreciably influenced by age.

Among the *H. pylori*-seronegative donors ($n = 26$), eight subjects (30.8%) were of Le (a+b–) phenotype (Table 2). This is significantly higher than in the *H. pylori*-seropositive group: 15 of 133 (11.3%); $\chi^2 = 5.2$, df 1, $P = 0.02$; OR = 3.5, 95% CI 1.2–10.5. It should be noted that the proportion of Le (a+b–)/non-secretors in the *H. pylori*-seropositive group was almost identical to those obtained in the population-based study in Estonia – 11.7% [10]. It was also true for *H. pylori*-seropositive individuals of Le (a–b+) and Le (a–b–) phenotypes (Table 2): 97 of 133 (72.9%) and 21 of 133 (15.8%) compared with the population-based data (70.0% and 16.9%, respectively) [10].

A significantly lower systemic immune response to *H. pylori* cell surface antigens, as defined by RAA values, was found in the *H. pylori*-seropositive individuals of Le (a+b–) blood group phenotype compared with those of two other phenotypes (Fig. 2). The subjects with the Le (a–b+) and Le (a–b–) phenotype showed very similar patterns.

Discussion

The data show that the Le (a+b–)/non-secretor phenotype is associated with a higher proportion of *H. pylori*-uninfected individuals among blood donors. Furthermore, the immune response of the *H. pylori*-seropositive individuals in this group is also lower than that of Le (a–b+)/secretors. It has been shown that the level of *H. pylori* IgG antibodies is related to the density of antrum mucosa colonization by *H. pylori* [13]. Thus, in contrast to some examples of an association of the non-secretor status with the susceptibility to a number of infectious diseases [14], the non-secretors appear to be more resistant to *H. pylori* infection.

The role of *H. pylori* adhesins in gastric mucosa colonization is not fully understood. Their interaction with the target tissue receptors, which are the normal constituents of the mucus, epithelial cells and connective tissue, and are present in every individual, could not explain the higher resistance to *H. pylori* infection in some individuals. Therefore, it is likely that this relative resistance is associated with some more polymorphic tissue receptors such as histo-blood-group antigens and their precursors and/or derivatives. In this context, a possible role of Le (b) antigen as a receptor for *H. pylori* attachment to gastric mucosa [1] is an intriguing idea.

However, we found that the Le (a–b–) phenotype group had a similar proportion of *H. pylori*-seronegative individuals, as well as low responders, as the Le (a–b+) phenotype group (Fig. 1). Moreover, no significant difference in the seronegativity rate was found between Le (b+) and Le (b–) donors, whereas the Le (a+) individuals had a significantly higher proportion of *H. pylori*-seronegative persons than the Le (a–) group. These findings suggest that the Le (a) or some other antigens associated with non-secretor status, but not the presence or absence of the Le (b) antigen, may be important in the natural resistance to *H. pylori*.

The expression of other carbohydrate epitopes on gastric mucins and on epithelial cell glycoconjugates has been shown to be dependent on secretor/non-secretor status [7,15]. In particular, the difference concerns the expression of T epitope (Galβ1-3 GalNAcα/β1) on type 3 mucin-type chains in non-secretors, exclusively [15]. In contrast, in secretors this epitope is further fucosylated

[16]. In addition to Le (b) antigen (Fuc1-2Galβ1-3(Fuc1-4)GlcNaAcβ1), expressed on type 1 or 2 chain glycoconjugates, the H-1 disaccharide (Fucα1-2Gal) was shown to be the minimal receptor for *H. pylori* adhesion [17]. Furthermore, a wide inter-individual quantitative variation in the expression of the Le antigens on mucus and epithelial cells may influence the adhesin–receptor interaction.

It should be noted that other researchers found no association between the prevalence of *H. pylori* infection and the proportion of non-secretors or Lewis blood group phenotypes in dyspeptic individuals and patients with peptic ulcers [4,18,19]. However, the prevalence of *H. pylori* infection in these patients is very high and one might expect alteration in the resistance mechanisms in these groups. In fact, our data also showed no appreciable alteration in the Le (a,b) phenotype distribution in the *H. pylori*-seropositive group of donors.

We have intentionally investigated an older group of blood donors. We suppose that, in the evaluation of the natural resistance mechanisms against *H. pylori*, a maximum of the *H. pylori* infection prevalence in the population should be chosen as a starting point. Under these circumstances, the individuals that remained uninfected should be considered as the most resistant population. Our data showed the significant prevalence of Le (a+b–) blood group phenotype in this group (Fig. 1). It is obvious that this maximum is achieved at different ages in different populations. In the Estonian population the prevalence of *H. pylori* infection is very high and reaches its plateau at about 30 years of age [8,9], with a seroprevalence rate similar to that observed in other countries for patients with chronic gastric diseases. Our data confirm these findings.

Höök-Nikanne *et al.* [20] studied a group of blood donors at 36–45 years of age and found no relation between *H. pylori* seropositivity rate and secretor/non-secretor status. These investigators also used the glycine extract of the *H. pylori* strain NCTC 11637, but other criteria for the seropositivity based on the end-point titre were employed. In addition, a much lower *H. pylori* seroprevalence rate (42%, vs. 83.6% in this study) and a higher proportion of non-secretors (20% vs. 11.7%) was demonstrated in the Finnish blood donors, making it difficult to compare with our findings. Our data are, however, in accordance with the findings of Mentis *et al.* [4] who revealed the bacteria by either culture or biopsy in 12.5% of Le (a+)/non-secretors (1 of 8) compared with 67% (28 of 42) in Le (b+)/secretors ($P < 0.01$) in patients with gastric ulcer.

In conclusion, individuals with Lewis (a+b–)/non-secretor phenotype showed a significantly higher proportion of *H. pylori*-seronegative results and seemed to be more resistant to *H. pylori* infection. The Lewis (a+b–) phenotype was also associated with a lower strength of immune response to *H. pylori* antigens as defined by RAA values in ELISA. Our findings support the idea that Le (a,b) histo-blood-group antigens are implicated in the mechanisms of the naturally occurring resistance to *H. pylori* infection and in the pathogenesis of *H. pylori*-associated diseases.

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III

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IgG antibodies to Lewis type 2 antigens in serum of *H. pylori*-infected and noninfected blood donors of different Lewis(a,b) blood-group phenotype

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Abstract

Individuals of the Le(b+)/secretor phenotype revealed a stronger natural immune response to Le(x) and Le(y) epitopes irrespective of *Helicobacter pylori* serologic status. In contrast, *H. pylori*-infected Le(b−) type individuals showed a significantly higher proportion of strong responders to Le(x) antigen compared with the *H. pylori*-uninfected subgroup. The data suggest that the immune response to Lewis type 2 determinants is related to both the *H. pylori* serologic status and the Le(a,b) phenotype of the host.

Keywords: *Helicobacter pylori*; Lewis antigen; Blood group antigen; Immune response

1. Introduction

The pathogenicity of microbes is closely related to their phenotypic diversity by which they can adapt to host microenvironments and evade the host immune response [6]. The majority of *Helicobacter pylori* strains was shown to express human blood group type 2 mono- and difucosylated oligosaccharide structures, i.e. Le(x) and Le(y) antigens [2,13,14]. This expression is related to the host Le(a,b) histo-blood group phenotype and positively associated with CagA status [15,16]. The immune response to these epitopes has been demonstrated in both *H. pylori*-infected and uninfected individuals [1] and *H. pylori*-infected individuals with a low level of anti-Le(x) antibodies were shown to be at a higher risk for gastric mucosa atrophy development [9] suggesting that an immune response to Le type 2 antigens may be related to the clinical outcome of the infection. The aim of this study was to explore if the immune response to Le(x) and Le(y) antigens is related to host Lewis(a,b) histo-blood group pheno-

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type and/or *H. pylori* serologic status of the individual.

2. Materials and methods

2.1. Subjects and samples

Blood samples were obtained from 87 blood transfusion donors (33 men, 43 women, median age 47.2, range 19–65 years) selected by Le(a,b) blood group phenotype (Table 1). Donors were not asked about the presence of abdominal complaints. The sera were separated after blood clotting and stored at −20°C until studied.

2.2. Lewis(a,b) phenotyping

Lewis(a,b) phenotyping of erythrocytes was carried out using anti-Le(a) and anti-Le(b) monoclonal antibody gel system (DiaMed, Switzerland) according to the instructions of the manufacturer.

2.3. H. pylori antigen preparation

A glycine cell surface antigen extraction of *H. pylori* strain NCTC 11637 was performed according to Logan and Trust [11]. The strain was grown on GAB-CAMP reference agar at 37°C for 2 days in plastic jars filled with a gas mixture of 5% O_2, 10% CO_2 and 85% N_2. A pool of five different preparations was used as an antigen in *H. pylori* ELISA.

2.4. H. pylori enzyme-linked immunosorbent assay (ELISA)

Serum samples were examined by ELISA as described earlier [10]. In brief, 96-well flat-bottom microtitre plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with 0.1 ml of antigen (5 μg ml^{-1}) per well and incubated at 4°C overnight. Alkaline phosphatase-conjugated goat anti-human IgG (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) and *p*-nitrophenylphosphate (Sigma, St. Louis, MO, USA) were used. A pool of human IgG (Kabi AB, Stockholm, Sweden) was placed in each ELISA plate as a positive control for 100 units, and *H. pylori*-seronegative reference serum was run as a negative control. Relative antibody activity values above 25 were regarded as positive.

2.5. Determination of serum Le(x) and Le(y) IgG antibodies by ELISA

Plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with synthetic Le(x) and Le(y) hapten-polyacrylamide conjugates (Synthesom, Munich, Germany; 20 mol% of carbohydrates) 2 μg ml^{-1} in sodium carbonate buffer 50 mM $Na_2CO_3/NaHCO_3$ and 0.02% NaN_3, pH 9.6 at 4°C, or 1% BSA in PBS (control wells) overnight. After washing three times with PBS (0.13 M NaCl, 8 mM NaH_2PO_4/KH_2PO_4, 3 mM KCl, 2 mM EDTA, pH 7.2)-0.05% Tween 20 the plates were blocked with 0.15 ml of 1% BSA in PBS for 1 h at room temperature (RT) and washed in PBS-Tween. Serum (100 μl) diluted 1:100 in PBS-Tween was added for 2 h at RT. The plates were then washed and bound IgG antibodies were detected with 100 μl of alkaline phosphatase-conjugated goat anti-human IgG (Gibco, BRL, Life Technologies, Gaithersburg, MD, USA) diluted 1:1000 in PBS-Tween. Following an incubation of 90 min at RT and washing, the plates were developed with *p*-nitrophenylphosphate (Sigma, St. Louis, MO, USA), 1 mg ml^{-1} in 1 M diethanolamine buffer, pH 9.8 for 30 min and the absorbance values at 405 nm were registered with Labsystem Multiscan MCC/340 (Finland). The optical density values of control wells were subtracted from the values of the wells coated with the Le conjugates and net OD × 100 unit values more than 15 were considered a strong response. Each serum was analysed in duplicate and determination of Le(x) and Le(y) antibodies in a given serum was performed in the same plate. Two reference sera from weak and strong responders were run in every plate to correct the inter-assay variations.

2.6. Statistical analysis

Fisher's exact test was used to compare the proportions. The means were compared with Student's *t*-test. The difference was considered to be significant when $P < 0.05$.

3. Results

Typical serum titration curves for IgG antibody to Le(x) and Le(y) antigens are shown in Fig. 1. A lower background and the best discrimination between weak and strong responders were obtained at a serum dilution 1:100 and a cut-off limit equal to 15 ODU.

A majority of the individuals showed an IgG immune response to the Le(x,y) epitopes. However, no appreciable differences between donors of different Le(a,b) phenotypes were observed when the data were analysed irrespective of *H. pylori* serologic status (Table 1). The only difference found was a lower proportion of high responders to the Le(y) epitope among donors of the Le(a+b−) and Le(a−b−) phenotypes compared with those of the Le(a−b+) type but the difference was significant only for individuals of the Le(a−b−) type subgroup ($P=0.02$). If the Le(a+b−) and Le(a−b−) groups of individuals were combined into an Le(b−) group and compared with donors of the Le(b+) type, the difference reached statistical significance for anti-Le(y) IgG response ($P=0.03$).

In contrast, *H. pylori*-seronegative and -seropositive donors showed different patterns of response (Fig. 2). The difference between *H. pylori*-infected and uninfected donors was observed mostly in individuals of the Le(b−) phenotype who showed a significantly higher proportion of strong responders to the Le(x) determinant among *H. pylori*-infected subjects ($P=0.04$). In addition, a significantly lower proportion of strong responders to the Le(x) antigen was found among *H. pylori*-seronegative donors of the Le(b−) phenotype compared with uninfected individuals of the Le(b+) type. The Le(b+)/secretors revealed a stronger response to Le(x) and Le(y) antigens in both the *H. pylori*-infected and uninfected donors. Some decrease in the level of Le(y) antibod-

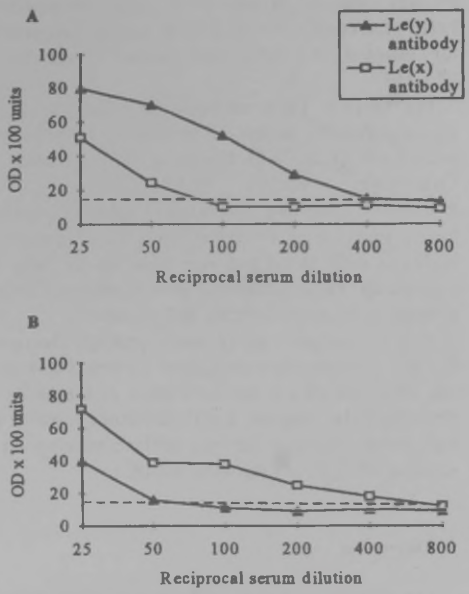

Fig. 1. Serum titration curves of Le(x) and Le(y) IgG for strong anti-Le(y) (A) and anti-Le(x) (B) responders. Wells were coated with Le(x) and Le(y) polyacrylamide conjugates 2 μg ml^{-1}, serum dilution 1:100. The OD_{405} values of control wells (1% BSA in PBS) were subtracted. Dotted line: cut-off limit (15 ODU).

Table 1
Proportion of strong responders to Le(x) and Le(y) antigens in blood donors of different Le(a,b) blood group phenotype irrespective of the *H. pylori* serologic status

Characteristic	Erythrocyte Lewis(a,b) phenotype			
	a+b−	a−b+	a−b−	b−[a]
Number of donors	23	45	19	42
Age (years, mean ± S.D.)	47.1 ± 10.1	46.5 ± 9.2	47.4 ± 7.2	47.4 ± 5.7
Males (%)	9 (39.1)	18 (40.0)	6 (31.6)	15 (35.7)
Number (%) of strong responders to Le(x)	15 (65.2)	35 (77.8)	12 (63.2)	27 (64.3)
Number (%) of strong responders to Le(y)	11 (47.8)	28 (62.2)	6 (31.6)[b]	17 (40.5)[c]

[a]Le(b−) combined group: Le(a+b−)+Le(a−b−) subjects.
[b]Le(a−b−) compared with Le(a−b+) group: $P=0.02$ (Fisher's exact test).
[c]Le(b−) compared with Le(b+) group: $P=0.03$ (Fisher's exact test).

ies was observed in sera of *H. pylori*-seropositive Le(b+)/secretors: 53% vs 80% of strong responders for infected and uninfected donors, respectively ($P = 0.07$).

The Le(y) to Le(x) antibody ratio (Le y/x ratio) was significantly higher in uninfected Le(b+)-type individuals (1.27 ± 0.6) compared with uninfected Le(b−)-type donors (0.68 ± 0.26; $t = 3.47$, $P < 0.001$) indicating that Le(b+) subjects usually have a higher level of natural Le(y) than Le(x) antibodies in their blood and vice versa for the Le(b−) type group. These differences were altered and insignificant in *H. pylori*-infected individuals.

Thus a relation of the *H. pylori* serologic status to the IgG immune response against Le type 2 epitopes was observed mostly for individuals of the Le(b−) phenotype. In contrast, Le(b+)/secretors revealed a high natural immune response to these antigens irrespective of *H. pylori* serologic status.

4. Discussion

The data show that the difference in the response to Le type 2 antigens between *H. pylori*-infected and uninfected donors was observed mostly in individuals of the Le(b−) phenotype who revealed a significantly higher proportion of strong responders to the Le(x) determinant among *H. pylori*-infected subjects. An appreciable though non-significant increase in the response to Le(y) antigen was also observed in this group.

It has been shown that gastric mucosa cells express Le(x) and Le(y) epitopes but immunohistochemically the topography is different, No Le(x) expression was found in the foveolar epithelial cells of either secretors or non-secretors whereas some amount of Le(y) can be detected in this area in secretors. However, both determinants were demonstrated in gastric glands irrespective of *Selse* status [12]. The higher immune response to the Le(x) antigen in *H. pylori*-infected subjects that we observed in this study may be related to the findings of Byrd et al. [5] who reported on aberrant expression of Le(x) in the surface epithelium of *H. pylori*-infected individuals. It would be interesting to know if this aberrant expression and an immune response to this antigen were associated with host Le or secretor phenotype and to investigate whether certain variants related to the clinical outcome of the infection.

Amano et al. [1] reported on the presence of natural antibodies to Le type 1 and type 2 antigens in the serum of every individual irrespective of *H. pylori* serologic status. They used synthetic conjugates of

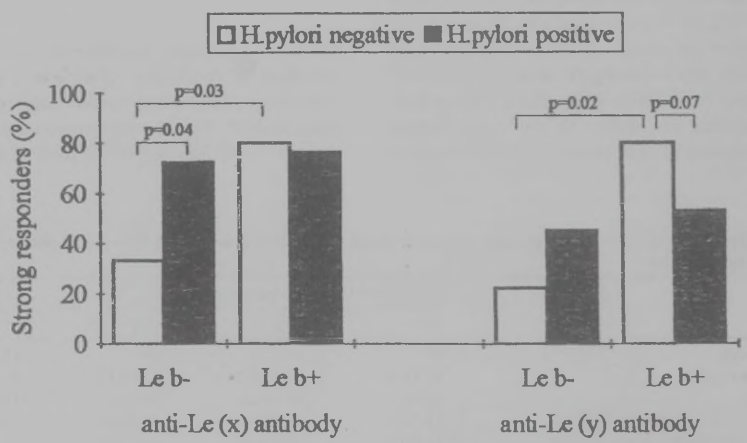

Fig. 2. IgG immune response to Le(x) and Le(y) synthetic antigens in relation to host Le(b−) and Le(b+) type and *H. pylori* serologic status. The proportion of strong responders (OD values × 100 more than 15) to Le(x) and Le(y) antigens was determined in *H. pylori*-seronegative ($n = 24$) and *H. pylori*-seropositive ($n = 63$) blood donors. *P* values as calculated by Fisher's exact test.

Le haptens with human serum albumin as antigens in ELISA but did not correlate the results with the host Le phenotype. We also did not find any difference in immune response to Le antigens between *H. pylori*-infected and uninfected individuals when the data were analysed in a similar way. This is because Le(b+)-type individuals showed a high proportion of strong responders to Le type 2 antigens irrespective of *H. pylori* serologic status (Fig. 2). A predominance of Le(b+)/secretors in a majority of populations makes it difficult to reveal an association between the immune response to Le(x,y) antigens and *H. pylori* status if the host Le phenotype was not taken into account.

The findings of Kuipers et al. [9] that *H. pylori*-infected subjects with a low level of Le(x) antibodies had a higher risk of gastric atrophy may also be related to our results though these authors did not tested the patients for Le phenotype. We found a rather low and similar proportion of low responders to Le(x) among *H. pylori*-infected donors of both Le(b−) and Le(b+) phenotype (27% and 20%, respectively). Thus the host Le type did not appreciably influence the immune response to this antigen in *H. pylori*-infected individuals. Since the levels of Le(x) and Le(y) antibodies in serum of *H. pylori*-uninfected individuals are inversely related, a prevalence of Le(y) over Le(x) antibodies is characteristic of Le(b+) individuals. In this context, an overrepresentation of Le(b+)-type individuals should be expected among subjects with a low level of Le(x) antibodies. On the other hand, uninfected individuals of the Le(b+) type had a high level of naturally occurring Le(x) and Le(y) antibodies. It is considered that natural antibodies, including those related to blood group antigens, might protect against autoimmunity or down-regulate the host T-cell response to microorganisms and the cytokine-induced inflammatory cascade [3,4,6]. In contrast, putatively 'immune' Le(x) antibodies which appear in response to *H. pylori* mainly in Le(b−)-type subjects may be more pathogenic in terms of their ability to induce inflammation. We have shown earlier that individuals of the Le(a+b−) phenotype demonstrated a lower IgG immune response to *H. pylori* antigens as compared with those of the Le(b+)/secretor phenotype [7]. A positive correlation between the density of colonisation by *H. pylori* and IgG immune response [8] indicates that humoral immune response may be not beneficial for the host. We might speculate that the individuals who had a low level of natural antibodies to Le type 2 antigens before *H. pylori* infection (i.e. Le(b−) subjects) but showed a higher immune response to these determinants after being infected with *H. pylori*, might be at a higher risk of gastric atrophy development.

In conclusion, the data suggest that the host Le(a,b) phenotype not only may be related to the expression of Lewis type 2 antigens by *H. pylori* [8] but also contributes to the host immune response to these determinants in both *H. pylori* -infected and uninfected individuals. It remains unclear, however, to what extent a natural or *H. pylori*-induced immune response to these epitopes might be beneficial or detrimental for the host and how it is related to the clinical outcome of the infection.

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IV

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ORIGINAL INVESTIGATION

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Immune response to a recombinant fragment of the CagA protein of *Helicobacter pylori* in blood donors and patients with gastric cancer: relation to ABO(H) blood group phenotype, stage of the disease and tumor morphology

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Abstract IgG immune response to CagA was evaluated by enzyme-linked imunosorbent assay (ELISA) using a recombinant fragment of CagA as antigen in 171 patients with gastric cancer and 298 blood donors to determine whether it could be related to the ABO(H) blood group phenotype, stage of cancer or tumor morphology. The CagA-ELISA showed a good specificity (93.5%) and sensitivity (88.5%) as compared with immunoblotting for blot CagA-negative and -positive donors. The *Helicobacter pylori* seropositive blood group A donors revealed the lowest proportion (37.6%) of strong responders to CagA: A<O (51.2%)<B (56.9%)<AB (62.5%). The proportion of strong responders to CagA was significantly lower among the *H. pylori*-seropositive patients with non-cardial cancer (35.4%) than in donors (48.8%). A significant suppression of immune response to CagA was found in the patients with advanced cancer. The proportion of CagA strong responders was higher at the first stage of gastric cancer in only blood group O and A individuals as compared with related controls. The overall CagA seroprevalence was not influenced by tumor histology. Thus, the IgG immune response to CagA is dependent on the ABO(H) blood group phenotype of the host and the stage of cancer. The host-dependent differences in the immune response to CagA may be more pronounced than those related to the putative disease-specific features of the *H. pylori* infection.

Key words *Helicobacter pylori* · Immune response · CagA · ABH blood groups · Gastric cancer

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Introduction

The *cagA* gene of *Helicobacter pylori* is the only nonconserved gene characterized to date which is present in about 60–70% of *H. pylori* strains [2, 7, 9]. The infection by *H. pylori* strains which possess the *cagA* gene or express the genomic product, a high molecular weight immunodominant protein (CagA), increases the risk of developing athrophic gastritis, peptic ulceration and gastric cancer [5, 16, 28]. The *cag* region is related to the phenotypic differences between type I pro-inflammatory (*cagA+ vacA+*) and type II (*cagA-vacA-*) *H. pylori* strains [7, 33]. The cagA-positive phenotype is associated with IL-8 expression in gastric epithelial cells [2, 11]. The CagA is highly immunogenic and the presence of serum anti-CagA antibodies strongly correlates with the *cagA*-positive status of the individual, and allows infection with *cagA*-positive strains to be detected serologically [10]. An enzyme-linked immunosorbent assay (ELISA) has been developed recently using recombinant fragments of CagA protein as antigen [5, 32].

Gastric epithelial cells, mucus and the *H. pylori* itself contain blood group antigen-related carbohydrate epitopes. An expression of Lewis antigens by *H. pylori* is related to the host Lewis phenotype [31] and positively associated with the presence of the cag pathogenecity island [13] and cagA status [30]. An over-representation of blood group A individuals among gastric cancer patients and the prevalence of blood group O subjects among patients with peptic ulcers were shown a long while ago [1, 8] but the basis for this association remains unknown. It has been reported recently that the leukocytes of blood group O donors revealed a stronger inflammatory response to *H. pylori* and released significantly higher amount of IL-6, TNF and nitric oxide than blood group A leukocytes [3]. All these findings suggest that (i) the host blood group phenotype may be associated with the infection by a particular *H. pylori* strain, and (ii) the immune response to *H. pylori* may vary depending on blood group phenotype of the host, thus predicting the

clinical outcome of *H. pylori* infection including gastric cancer.

We report here that immune responses to CagA, as determined by ELISA using a recombinant fragment of CagA as antigen, are related to the ABO(H) blood group phenotype in both *H. pylori*-seropositive blood donors and cancer patients, and that it is tumor stage-dependent and suppressed in the patients with advanced gastric cancer.

Material and methods

Subjects and samples

Serum samples were obtained before any treatment from 171 patients with gastric cancer admitted consecutively to the Estonian Cancer Center with histologically verified gastric carcinoma (106 men, 65 women, median age 62 years, range 40–75 years). Tumor staging (see Fig. 2) was based on histopathological (pTNM) classification and morphology was diagnosed according to the system of Lauren [19] as intestinal ($n = 97$) and diffuse ($n = 74$) type of tumor growth. The cancer site was cardial in 19 patients (11%) and all these tumors except one were of intestinal type. The controls were 298 randomly selected blood transfusion donors (117 men, 181 women, median age 57 years, range 40–72 years). Donors were not asked about the presence of abdominal complaints. The sera were separated after blood clotting and stored at –20 °C until studied.

Antigen preparation

A glycine cell surface antigen extraction of *H. pylori* strain NCTC 11637 (CagA and VacA positive) was performed according to Logan and Trust [22]. The strain was grown on Gonococcal agar base/Campylobacter (GAB-CAMP) reference agar at 37 °C for 2 days in plastic jars filled with a gas mixture of 5% O_2, 10% CO_2 and 85% N_2. A pool of nine different preparations was used as antigen in *H. pylori* ELISA and immunoblotting.

A recombinant fragment (37.5 kDa) coding for the immunodominant region of the 128-kDa protein (CagA) was expressed as a fusion protein in *Escherichia coli*, purified as described [32] and used in CagA-ELISA.

ELISA *H. pylori*

Serum samples were examined by ELISA as described earlier [14, 20]. In brief, 96-well flat bottom microtiter plates (Maxi Sorp, Nunc, Roskilde, Denmark) were coated with 0.1 ml antigen (5 µg/ml) per well and incubated at 4 °C overnight. Alkaline phosphatase-conjugated goat anti-human IgG (Gibco BRL, Life Technologies, Gaithersburg, Md.) and *p*-nitrophenylphosphate (Sigma, St. Louis, Mo.) as substrate were used. A pool of human IgG (Kabi AB, Stockholm, Sweden) was placed in each ELISA plate as a positive control for 100 units and *H. pylori*-seronegative reference serum was run as a negative control. Relative antibody activity (RAA) values were calculated according to Blomberg et al. [6] to eliminate interassay variations. RAA values ≤25 were regarded as negative. The specificity of the test has been reported to be 98% with a sensitivity of 89.7% [21].

SDS-PAGE and immunoblotting

SDS-PAGE was performed using a 12% polyacrylamide separating gel according to Laemmli [18]. A 1-mm gel (6×10 cm) was loaded with *H. pylori* glycine extract (µg protein per gel) and protein molecular weight standards (Merck, Darmstadt, Germany). After separation and blotting [14], the nitrocellulose membrane was saturated as described [29] and, after drying, cut into strips. The strips were washed and incubated with patients sera (1:76), overnight at 4 °C under agitaton. One strip was incubated with standard IgG in each plate as a positive control. The strips were washed and incubated for 2 h at 4 °C with peroxidase labelled rabbit anti-human IgG (Gibco) and developed with 3-amino-9-ethylcarbazol (Sigma). A positive reaction was defined as the presence of a specific CagA band of 120 kDa on the blots.

CagA-ELISA

CagA-ELISA was performed as described previously [32] with slight modifications. Flat-bottom polystyrene plates (Maxi Sorp, Nunc) were coated with 100 µl/well of the purified recombinant antigen solution (1.25 µg/ml). An alkaline-phosphatase-conjugated goat anti-human IgG and p-nitrophenyl phospate as substrate were used. The cut off limit equal to an absorbance of 0.42 was determined on the basis of the investigation of 26 *H. pylori*-ELISA and CagA blot-negative and of 46 CagA blot-positive blood donors sera. This cut off value gave the best discrimination between the groups, and absorbance values greater than 0.42 were regarded as a positive reaction (strong responders). Two reference serum from CagA-seropositive and CagA-negative donors, respectively, were included in each plate as internal standards to correct the inter-assay variations. A sensitivity of 96.2% and a specificity of 96.6% have been reported for this assay as compared with Western blot [32].

ABO(H) phenotyping

ABO(H) phenotyping of erythrocytes was carried out with anti-A and anti-B monoclonal antibodies (MonoCarb AB, Sweden).

Statistical analysis

Statistical comparisons between groups were performed by chi-square tests. The difference was considered to be significant when the *P* value was less than 0.05. Odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated to measure an association between the parameters studied.

Results

A group of CagA-positive and -negative blood donors ($n = 72$), determined by immunoblotting, were further tested by CagA-ELISA. A good correlation between two tests was found. Only 3 of 46 blot-positive individuals showed a negative response in CagA-ELISA and 3 of 26 CagA blot-negative donors were positive by CagA-ELISA. Thus, the sensitivity and specificity of CagA-ELISA as compared with blot was 93.5% and 88.5%, respectively.

The overall *H. pylori*-seropositivity rate as evaluated by conventional ELISA was very similar in donors (246 of 298; 82.5%) and in patients with non-cardial gastric cancer (130 of 152; 85.5%). The blood group phenotype distribution in donors and cancer patients for both the cardial and non-cardial tumors is shown in Table 1. The distribution of blood donors by ABO(H) blood group phenotype did not differ significantly from that found in the population-based study in Estonia [24]. However, a significant increase in the prevalence of blood group A individuals was observed in patients with non-cardial tumors as compared with donors (45.4% vs 35.2%; $\chi^2 = 3.96$,

Table 1 ABO(H) blood group phenotype distribution among blood donors and patients with gastric cancer. Blood group distribution in the Estonian population: blood group O 33.8%, A 35.3%, B 23.7% and AB 7.2% [24]. The values in parentheses represent % of individuals with blood group O, A, B or AB in each group

	n	O	A	B	AB
Blood donors:	298	104 (34.9)	105 (35.2)	61 (20.5)	28 (9.4)
Gastric cancer patients:					
non-cardial tumors	152	38 (25.0)	69 (45.4)*	36 (23.7)	9 (5.9)
cardial tumors	19	8 (42.1)	4 (21.05)	4 (21.05)	3 (15.8)
All patients	171	46 (26.9)	73 (42.7)	40 (23.4)	12 (7.0)

* Significantly different from related donor group: $\chi^2 = 3.96$, $P = 0.046$, odds ratio 1.5 (95% confidence interval 1.0–2.3)

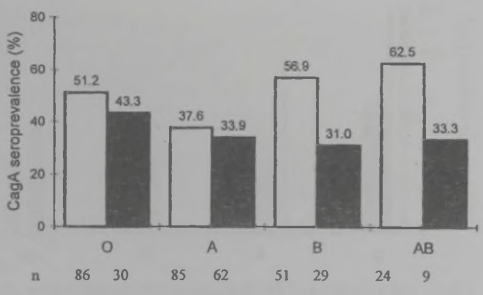

Fig. 1 The CagA seroprevalence in *Helicobacter pylori*-seropositive non-cardial cancer patients and controls by ABO(H) blood group phenotype (*open bars* blood donors, *closed bars* gastric cancer patients). The significant differences as calculated by chi-square test: blood group A donors vs blood group B donors, $\chi^2 = 4.0$, $P = 0.045$, OR = 2.2, 95% CI 1.0–4.7; blood group A donors vs blood group AB donors, $\chi^2 = 3.7$, $P = 0.052$, OR = 2.8, 95% CI 1.0–7.8; blood group B donors vs blood group B cancer patients, $\chi^2 = 3.96$, $P = 0.046$, OR = 2.9, 95% CI 1.0–8.6

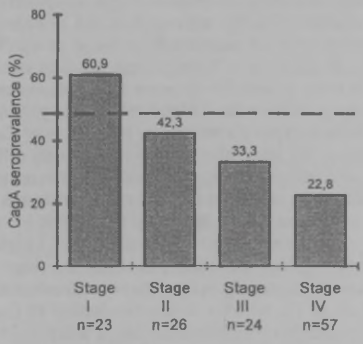

Fig. 2 The CagA seroprevalence in *H. pylori*-seropositive patients at different stages of gastric cancer. Significant differences as calculated by chi-square test: patients: stage I vs stage IV, $\chi^2 = 9.0$, $P = 0.003$, OR 5.3 (1.7–17.2), stage I+II vs stage III+IV, $\chi^2 = 7.3$, $P = 0.007$, OR 3.0 (1.3–6.8); donors vs patients at stage IV, $\chi^2 = 11.6$, $P = 0.0006$, OR 3.2 (1.6–6.6); donors vs patients at stage III+IV, $\chi^2 = 12.1$, $P = 0.0005$, OR 2.7 (1.5–4.9). *Horizontal dotted line* shows a proportion of CagA-seropositive blood donors

$P<0.05$). An over-representation of blood group A subjects was mostly related to the patients with diffuse-type tumors (32 of 63, 50.8%; $\chi^2 = 4.7$, $P = 0.03$, OR = 1.9, 95% CI = 1.1–3.4). In contrast, a lower proportion of blood group O individuals in patients with non-cardial cancer was noted.

The differences in the *H. pylori* seroprevalence among the individuals of different ABO(H) blood group phenotype were non-significant: 82.7%, 78.1%, 83.6% and 85.7% for donors of blood group O, A, B, and AB, respectively, and 78.9%, 89.9%, 80.6% and 100% for the related group of patients. There was also no significant difference between patients and controls. Patients possessing the blood group A antigen (i.e., A+AB) showed a tendency ($P = 0.08$) for a higher *H. pylori* seroprevalence (91.0%) compared with blood group O+B individuals (79.7%).

In contrast, using CagA-ELISA, 46 of 130 (35.4%) *H. pylori*-seropositive non-cardial cancer patients and 120 of 246 (48.8%) blood donors were CagA strong responders, which is significantly lower than for patients with gastric cancer: $\chi^2 = 5.7$, $P = 0.02$; OR = 1.7, 95% CI (1.1–2.8). Only 1 of 22 (4.5%) cancer patients and 6 of 52 (11.5%) blood donors, who were negative in conventional *H. pylori*-ELISA, also showed a positive anti-CagA immune response. Only the *H. pylori*-seropositive patients and controls were further analyzed in relation to the other parameters studied.

Appreciable differences in the CagA-seropositivity rate were found in blood donors of different ABO(H) blood group phenotype. The blood group A individuals revealed the lowest proportion of strong responders to the CagA compared with other phenotypes, especially with subjects of B and AB blood group, where the CagA seroprevalence was significantly higher (Fig. 1). The proportion of CagA-seropositive donors increased from blood groups A to AB in the order: A<O<B<AB. However, no remarkable differences between individuals of different blood groups, except a slightly higher percentage of CagA responders among blood group O subjects (43.3%), were noted in cancer patients if the data were analyzed regardless of the disease stage. Blood group B patients showed significantly lower proportion of CagA responders than blood group B donors.

A significant decrease in the proportion of CagA-seropositive cancer patients was found from disease stage I to stage IV (Fig. 2). An appreciably higher, though insignificant, CagA seroprevalence was observed in patients at the first stage of cancer as compared with controls: OR = 2.1; 95% CI (0.6–8.5). The difference between donors and the patients at stage IV or stage III+IV, as well as between early (I+II) and late stages of cancer was significant.

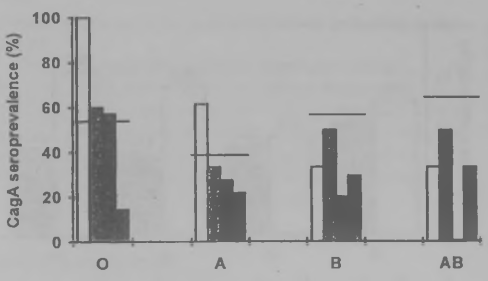

Fig. 3 The proportion of the CagA-seropositive gastric cancer patients by the disease stage and ABO(H) blood group phenotype. Bars – the patients with non-cardial gastric cancer at stage I, II, III and IV, respectively. Horizontal line is a proportion of CagA-seropositive individuals among blood donors of related blood-group phenotype. Blood group O cancer patients at stage I+II vs. patients at stage IV $\chi^2 = 4.4$, $p = 0.03$, OR = 6.7, 95% CI (1.1–66.4). Blood group A cancer patients at stage I vs. patients at stage IV $\chi^2 = 4.1$, $p = 0.04$, OR = 5.8, 95% CI (1.0–34.9)

A stage-dependent decrease in CagA-seropositivity rate with a drastic fall in the response to CagA at stage IV was observed only in the individuals of blood group O and A (Fig. 3). In addition, a higher CagA seroprevalence was found in these groups for the stage I patients as compared with donors of the related blood group. The patients of B and AB phenotypes showed a low proportion of CagA responders at any stage of cancer. No significant difference in the CagA seroprevalence was noted between the patients with diffuse or intestinal type of tumor, although it was higher in the last group: 30.2% vs 40.3%; OR = 1.6, 95% CI (0.7–3.5). As compared with blood donors, a higher proportion of CagA-positive subjects at the first stage of cancer was revealed mainly in the patients with intestinal-type tumors (8 of 12, 66.6%). A similar decline in CagA response in patients with advanced cancer was noted for both groups. It is notable that patients with the diffuse type of cancer showed the highest proportion of blood group A individuals (32 of 63, 50.8%) and at the same time the lowest proportion of CagA-seropositive subjects in this group (7 of 32, 21.9%) as compared with blood group A patients with the intestinal-type tumors (13 of 29, 44.8%; OR = 2.9; 95% CI 0.8–10.3). Thus, the blood group distribution among the individuals studied may appreciably influence the CagA-seropositivity rate.

All 19 cardial tumors except 1 were of intestinal type. A lower *H. pylori* seroprevalence was noted in the patients with cancer of the cardia (14 of 19, 73.7%) as compared with non-cardial tumors (130 of 152, 85.5%; OR = 2.1, 95% CI 0.6–7.1), and only 3 of 14 (21.4%) *H. pylori*-seropositive patients with cardial tumors were CagA seropositive. However, a lower *H. pylori* and CagA seroprevalence in this group may be because of all the patients with cardial tumors were at III and IV stage of cancer.

Discussion

The data indicate that CagA-ELISA using a recombinant fragment of CagA as antigen shows a good correlation with immunoblotting for CagA-positive and -negative sera from blood donors. It is well known that immunoblotting is a more sensitive method but the ELISA permits a quantitative evaluation of the reaction, whereas blotting permits only a semiquantitative one in which an inappropriate estimation can not be excluded. Despite some limitations, the CagA-ELISA seems to be a reliable assay for the evaluation of the immune response to CagA and for detection of the CagA status, at least in benign gastric disorders.

A rather low CagA seroprevalence among the *H. pylori*-seropositive blood donors (~50%) was observed in this study and even lower proportion (~35%) of cagA-seropositive gastric cancer patients was demonstrated. Previously, we reported [14] a lower proportion of CagA-seropositive individuals, detected by immunoblotting, among *H. pylori*-seropositive cancer patients compared with blood donors (55% and 69%, respectively), although the percentages were higher for both groups compared with CagA-ELISA in this study. However, we have shown that the stage of cancer is a crucial factor for the detection of CagA antibodies in CagA-ELISA. In addition, the anti-CagA response is related to the ABO(H) blood group phenotype of the host. The *H. pylori*-seropositive donors of blood group A showed the lowest proportion of CagA-seropositive individuals. This suggest that that blood group A individuals more often seem to be the 'weak CagA responders'.

It is possible that the frequency of CagA+ *H. pylori* strains, as defined by CagA-ELISA, may be underestimated in the present study because some individuals with a lower response (absorbance <0.42) were considered as CagA negative. Our data, however, are very similar to the findings of Kuipers et al. [16], who found 41% of CagA-seropositive subjects among the patients with chronic gastritis, using another recombinant fragment of CagA as antigen in ELISA. Another possibility to explain a low response to CagA in some individuals might be that the mixed *H. pylori* infections with CagA-positive and negative strains are present in some cases with a prevalence of the latter. It has been shown that up to 25% of the mixed *H. pylori* infections may be observed [12]. If so, *cagA* genotyping of *H. pylori* culture obtained from a single biopsy may be inappropriate due to a possibility of missing a CagA-positive strain that may be present in another places of the mucosa. A global assay such as serology would be more informative in this case. On the other hand, a lower response of some *H. pylori*-seropositive cancer patients to the CagA may simply reflect a prevalence of CagA-negative strains. In this context it would be interesting to evaluate whether the strength of the immune response to the CagA is more related to the quantity of CagA produced or to the genetic variations in the host response. The differences we found in the response to CagA between donors of different ABO(H) blood group phenotype support the

last possibility. It can not be excluded that blood group A subjects possess some kind of resistance against CagA-positive *H. pylori* strains. Blood group-related carbohydrate epitopes of both the *H. pylori* and the host have been shown to influence the adhesion properties of *H. pylori* (H-1, H-2, Le b) [4, 13] as well as the host immune response to the microorganism [5, 17, 27]. Therefore, it is likely that the host blood group phenotype might predict the infection with the *H. pylori* strain of particular phenotype.

Our data suggest that the overall CagA seroprevalence evaluated irrespective of blood group phenotype does not reflect the CagA status in cancer patients adequately, especially in countries with a lower proportion of CagA-positive strains. It is notable that patients with blood group O and A showed a higher proportion of CagA-positive subjects only at the first stage of cancer compared with donors with related blood group phenotypes. Therefore, the real CagA-seroprevalence may be estimated only at the very early stages gastric cancer in which the situation may be approximated to be similar to that at the onset of neoplasia. Many secondary phenomena observed in the patients with advanced cancer, such as an altered microenviroment due to aberrant glycosylation, higher pH, microbial overgrowth, and tumor-induced immunosuppression, may appreciably modulate the host-*H. pylori* relationship. In addition, it has been shown that the strength of an association between the CagA status and gastric cancer may vary appreciably between different populations [23, 26]. No such association could be expected in a population in which the proportion of CagA expressing strains reaches 90% or more, as in Japan [23]. We believe that the worldwide variations in ABO(H) blood group phenotype distribution may influence the CagA-seropositivity rate obtained by different investigators.

We found no significant difference in the immune response to CagA between the patients with diffuse and intestinal type of tumor growth. It is known that diffuse-type tumors are more often developed on the background of a relatively normal mucosa in younger males with blood group A phenotype [8, 25]. We also found an over-representation of patients with blood group A in diffuse-type cancer patients. In spite of the observation that blood group A donors are more often CagA seronegative, the blood group A cagA-seropositive individuals are over-represented among the patients with early gastric cancer (Fig. 3). It looks like some kind of 'enrichment' of blood group A CagA-seropositive individuals occurs among these patients. We might speculate that blood group A CagA-seropositive individuals are persons who are at a higher risk for cancer. It remains unclear, however, to what extent these associations relate to the host or to a particular microbial strain.

We can conclude that the immune response to the CagA as detected by CagA-ELISA is associated with ABO(H) blood group phenotype. Blood group A donors revealed the lowest proportion of strong responders to the CagA. Gastric cancer patients of blood group O and A had a higher proportion of CagA-seropositive individuals at the first stage of disease as compared with the blood group-related controls. A suppression of the immune response to CagA at the advanced stages of cancer was observed. Our findings suggest that several factors, such as blood group phenotype or other genetic traits of the individual, may influence the host immune response to *H. pylori* antigens and play a role in determining a disposition for an individual to be infected with a particular *H. pylori* strain. Before considering that inter-strain differences may be important in determining the clinical outcome of the *H. pylori* infection, it will be necessary to further evaluate whether this could be explained by inter-individual differences in response to *H. pylori* rather than among *H. pylori* strains.

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Helicobacter pylori ja CagA seroloogiline staatus gastroduodenaalse patoloogiaga haigetel: seos peremeesorganismi ABO(H), Lewisi fenotüübi ning sekretoorse staatusega

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Helicobacter pylori, immuunvastus, peremeesorganismi tegurid, veregrupid

Epidemioloogilised uuringud on näidanud, et Eestis on suur osa täiskasvanud elanikkonnast nakatunud *H. pylori* infektsiooni, kuid senini pole *H. pylori* infektsiooni kliiniline tähendus ja patogenees lõplikult selge. Selles uurimuses on selgitatud *H. pylori* esinemissagedust erinevate maohaiguste korral ja doonoritel, samuti peremeesorganismist tulenevate immunoloogiliste tegurite mõju virulentsemate *H. pylori* tüvedega nakatumisel.

Helicobacter pylori (*H. pylori*) infektsioon on seotud kroonilise gastriidi, peptilise haavandi ning maokasvajate (vähk, MALT-lümfoom) tekkimisega (1–3). Umbes 80% Eesti täiskasvanud elanikkonnast on nakatunud sellesse infektsiooni; see on ligikaudu kaks korda rohkem kui Skandinaavias ja Lääne-Euroopas (4, 5). Siiani pole leitud selliseid *H. pylori* tüvesid, mis oleksid mao haavandtõve või vähi otseseks tekitajaks. Küll aga on kindlaks tehtud mitmeid seoseid mõnede *H. pylori* geenide, eriti *cagA* (tsütotoksiiniga seotud geen A) ja *vacA* (vakuoliseerivat tsütotoksiini kodeeriv geen) virulentsustegurite ning peptilise haavandi ja gastriidi agressiivsuse vahel (1, 6–8). Uurimuste tulemused on siiski vasturääkivad, eriti populatsioonides, kus suur osa elanikkonnast on nakatunud *H. pylori* infektsiooni ning paljud selle nakkuse virulentsemate tüvedega (9–11). Paljudel CagA (*cagA* geeni produkt) positiivsetel isikutel ei ole mingeid kaebusi ja ka mao limaskestas puuduvad ilmsed patoloogilised muutused. Sageli on patsiendid nakatunud mõlema *H. pylori* tüvega, nii CagA+ kui ka CagA– tüvega (12). Selle probleemi prioriteetseks ülesandeks on uute kriteeriumide otsing *H. pylori* infektsiooni kliiniliste tagajärgede ennustamiseks ning selle nakkusega seotud haiguste patogeneesi väljaselgitamiseks.

Peremeesorganismi ja mikroobi omavahelistes suhetes on tähtis roll süsivesikantigeenidel (13). See puudutab kõigepealt väga polümorfset ABO(H)-süsteemi ja Lewisi antigeene ning nende derivaate, mis on laialdaselt esindatud mao mutsiinidel ja limaskesta rakkude glükokonjugaatidel, s.t on *H. pylori* vahetuks elukeskkonnaks. Erinevate süsivesikantigeenide ekspressioon on seotud *H. pylori* kolonisatsioonitiheduse ja põletiku astmega maos ning süsivesik- ja *H. pylori* antigeenidele suunatud immuunreaktsioonidega (5, 14–16). Peremeesorganismi ABO(H) ja Lewisi (Le) fenotüübi (raku või organismi jälgitav tunnus) ning sekretoorse staatuse võimalikku seost *H. pylori* teatud genotüübiga uuritud ei ole. Sekretoorne staatus (*Se/se* staatus) on võime eritada või mitte eritada sekreetidega (sülg, maomahl jt) vere ABO(H) grupiantigeene. CagA staatus on selles probleemis mugavaks mudeliks, sest see on üks kõige spetsiifilisemaid ja läbiuuritumaid *H. pylori* virulentsustegureid, mida on kerge ka seroloogiliselt määrata.

Selles töös uurisime ABO(H) ja Lewisi fenotüübi ning peremeesorganismi *Se/se* staatuse mõju *H. pylori* infektsiooni ja *H. pylori* CagA-positiivsete tüvede esinemissagedusele veredoonorite, maovähi ja mao healoomuliste haigustega haigete seas.

Tabel 1. *H. pylori* ja CagA seropositiivsus uuritud rühmades

		H. pylori +		Cag A +		Vanus (mediaan)
	n	n	%	n	%	
Doonorid	182	144	79,1	85	59,0	52,3
Maovähk	254	188	74,0	138	73,4**	59,7
I staadium	44	39	86,4	32	72,7	61,6
II staadium	42	34	80,9	27	79,4	56,7
III staadium	59	43	72,9	34	79,0	59,2
IV staadium	109	72	66,0*	45	62,5***	60,1
Healoomulised halgused	204	166	81,4	138	83,1**	55,4
Maohaavand	45	34	75,5	31	91,2**	56,5
Kaksteistsõrmiksoole-haavand	87	75	86,2	61	81,3**	54,0
Gastriit ilma atroofiata	31	25	80,6	23	74,2	53,0
Atroofiline gastriit	41	32	78,0	23	56,1	57,0

Statistiliselt usaldusväärsed erinevused arvutati hii-ruut(χ^2)-testiga.
* Võrreldes seropositiivsete isikute protsendiga I + II staadiumis vähihaigete ja kaksteistsõrmiksoole-haavandiga haigete seas;
** statistiliselt usaldusväärne erinevus võrreldes CagA-seropositiivsete doonoritega;
*** statistiliselt usaldusväärne erinevus võrreldes I, II ja III staadiumis maovähihaigete ($p = 0,002$) ja peptilise haavandiga patsientidega ($p < 10^{-6}$).

Uurimismaterjal ja -meetodid

Tehtud katsete maht ja iseloomustus on esitatud tabelites 1-3 ja joonisel 1. Uurimisobjektiks olid perifeerne veri, sülg, operatsiooni- ja gastrobiopsia materjal (kasvajakoe ja mao limaskesta proovid). Maovähi diagnoos kinnitati kõikidel juhtudel histoloogiliselt, peptilise haavandi diagnoos gastroskoopiliselt. Kroonilise gastriidi haiged jagati kahte rühma: atroofilise gastriidiga ja gastriit ilma atroofiata. Patohistoloogilist materjali hinnati morfoloogiliselt Sydney süsteemi järgi: uuriti mao antrumist ja korpusest võetud 1-2 limaskesta proovi. Atroofilise gastriidina hinnati ükskõik millisest mao osast võetud proovis leitud mõõdukat või tugevat atroofiat. Kõik uuritavad olid 40aastased või vanemad.

H. pylori ja CagA staatus määrati immunoblottingu ja immunoensüümmeetodil, mida on varem kirjeldatud (5, 16). Anti-*H. pylori* IgG antikehade tase väljendati antikehade suhtelise aktiivsusena - RAA (*relative antibody activity*) ja RAA <25 loeti seronegatiivseks tulemuseks. Uuritavad, kelle RAA langes vahemikku 26-35, nn *grey zone*, jäeti uuringust välja. Kontrollseerumitena kasutati *H. pylori* ja CagA-negatiivseid ja -positiivseid seerumeid. ABO(H) ja Lewisi fenotüüp määrati erütrotsüütide järgi, kasutades monokloonseid antikehi (5).

Sekretoorset staatust (*Se/se*) määrati immunoensüümmeetodil (17) H-antigeeni sisalduse järgi süljes (stimuleerimata sekretsioon) kasutades H-antigeen-spetsiifilist biotinüleeritud lektiini *Ulex Europeus* UEA I (Sigma, St Luis, MO) ning tulemust hinnati värvusreaktsiooni järgi, mis tekkis avidiiniga konjugeeritud peroksüdaasi (Sigma) reageerimisel substraadi O-fenüleendiamiiniga. Süljega sekreteeritava H-antigeeni tase (OD-väärtus) eelnevalt erütrotsüütide järgi testitud sekreetoritel [Le(a-b+)] oli kõikidel juhtudel ($n = 16$) 3-20 korda kõrgem kui mittesekreetoritel [Le(a+b-)]. Väiksemaid OD-väärtusi kui 0,25 (keskmine OD mittesekreetorite rühmas ($n = 12$) ± 2txSE, $p = 0,05$ juures) hinnati kui H-antigeeni sekretsiooni puudumist (mittesekreetorid). Igas katses kasutati sisemise standardina kontrollseerumeid (üks sekreetori ja üks mittesekreetori seerum). Andmeid töödeldi statistiliselt kasutades χ^2 ja Fisheri testi (väikeste gruppide jaoks).

Tulemused

H. pylori ja CagA seroloogiline staatus

(vt tabel 1)

H. pylori seropositiivsete isikute hulk uurimisrühmades oluliselt ei erinenud. Ainult maovähi IV staadiumis vähenes *H. pylori* seropositiivsus statistiliselt usaldusväärselt võrreldes vähi varasemate staadiumide (I-II), doonorite ($p = 0,02$)

Tabel 2. *H. pylori* ja CagA staatus ABO-veregruppide järgi

		H. pylori staatus				CagA staatus*				
	n / %	O	A	B	AB	O	A	B	AB	kõik n / %
Doonorid	n = 182	73	60	28	21	60	44	24	16	144
	HP+ (n)	62	44	22	16	42	17	16	10	85
	HP+ (%)	84,9	73,3	78,5	79,1	70,0	38,6**	70,8	62,5	59,0
Maovähk	n = 254	79	88	53	34	61	46	43	38	188
	HP+ (n)	61	63	42	22	45	32	34	27	138
	HP+ (%)	77,2	71,5	79,2	64,7	73,7	69,5	79,0	71,0	73,4
Hea-	n = 116	35	36	29	16	27	32	27	12	98
loomulised	HP+ (n)	31	29	25	13	24	21	22	9	76
haigused	HP+ (%)	88,5	80,5	86,2	81,2	88,8	65,6***	81,4	75,0	77,5

HP+: *H. pylori* seropositiivsed isikud.
* CagA staatust testiti ainult *H. pylori* positiivsetel isikutel: doonorid – 144, vähihaiged –138, healoomuliste haigustega grupp – 98.
** Usaldusväärselt erinev võrreldes O- ja B-veregrupi doonoritega ning samuti A-antigeeniga (A + AB veregrupp) ning ilma A-antigeenita (O + B veregrupp) isikute vahel.
*** Statistiliselt usaldusväärne võrreldes O-veregrupiga patsientidega (p = 0,025) ning A-antigeeniga (A + AB) ja A-antigeenita (O + B) isikute vahel (Fisheri test).

ning kaksteistsõrmiksoole-haavandi haigetega (p <0,01).

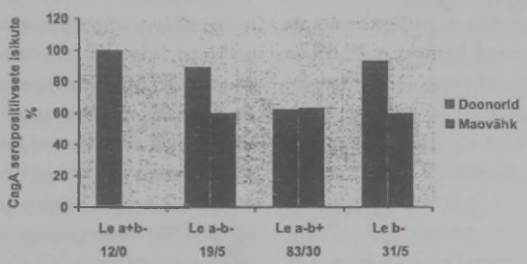
H. pylori seropositiivsete doonorite seas oli CagA seropositiivsuse tase usaldusväärselt madalam võrreldes nii maovähihaigete (p = 0,008) kui ka mao healoomuliste haigustega patsientidega (p <0,001). Kõige rohkem CagA-positiivseid isikuid oli maohaavandihaigete hulgas (45st 31; 91,2%). Maovähihaigete grupis oli see näitaja ligilähedane kroonilise mitteatroofilise gastriidiga haigete omale, kuid usaldusväärselt väiksem kui peptilise haavandiga haigetel. CagA-seropositiivsete isikute arv väheneb kasvaja arengu viimases staadiumis (62,5%) ning atroofilise gastriidiga haigete hulgas (56,1%).

ABO(H) fenotüübi seos *H. pylori*ga ja CagA staatusega (vt tabel 2)

Kindlat seost *H. pylori* infektsiooni esinemissageduse ja ABO(H) fenotüübi vahel ei leitud. A-veregrupiga doonorite ja healoomuliste haigustega patsientide seas oli küll vähem *H. pylori*'ga nakatunud isikuid, kuid erinevused ei olnud statistiliselt usaldusväärsed. Statistiliselt usaldusväärsed erinevused leiti CagA staatuse suhtes. CagA-positiivsete juhtumite arv oli kõige väiksem A-veregrupiga doonorite seas (p = 0,0028) võrreldes O- ja B-veregrupi või A+ ja A- fenotüübiga doonorite ($\chi^2 = 7,4$; p = 0,006), aga samuti healoomuliste haigustega haigete seas (p = 0,04). Maovähihaigete rühmas olid need erinevused vähem märgatavad.

Inimese Le(a,b) fenotüüp ja CagA seroloogiline staatus

Inimese Le fenotüübi ja CagA seropositiivsuse võimaliku seose uurimise tulemused on esitatud joonisel 1.



Joonis 1. CagA seroloogiline staatus veredoonoritel ja maovähihaigetel sõltuvalt inimese Lewisi fenotüübist.

Tabel 3. CagA seroloogiline staatus *H. pylori*'ga nakatunud ja mao healoomuliste haigustega isikutel sõltuvalt nende sekretoorsest (Se/se) staatusest

Uuritud rühmad	Sekreetorid (Se)		Mittesekreetorid (se)		Kokku	
	CagA+	CagA–	CagA+	CagA–	CagA+	CagA–
Maohaavand	19 (90,5%)	2	2 (66,7%)	1	21 (87,5%)	3
Kakstelstsõrmiksoole-haavand	23 (76,7%)	7	12 (80,0%)	3	35 (77,8%)	10
Gastrilt ilma atrooflata	14 (73,7%)	5	1 (50,0%)	1	15 (71,4%)	6
Atroofiline gastrilt	22 (78,6%)	6	2 (66,7%)	1	24 (77,4%)	7
Kokku	78 (79,6%)	20	17 (73,9%)	6	95 (78,5%)	26

Sulgudes CagA-positiivsete juhtude protsent.

Le(a+b–) ja Le(a–b–) fenotüübiga doonorite seas oli usaldusväärselt kõrgem CagA seropositiivsus võrreldes Le(a–b+) fenotüübiga isikutega (p = 0,039 ja p = 0,019 vastavalt Le(a+b–) ja ühendatud Le(b–) grupile). Esimesed kaks fenotüüpi omavahel selles suhtes oluliselt ei erinenud.

Uuritud 35 maovähihaige hulgas ei olnud ühtegi Le(a+) fenotüübiga isikut. Le(a–b+) fenotüübiga patsientide rühmas oli 63,3% CagA-seropositiivseid isikuid, mis on võrreldav sama fenotüübiga doonoritega (62,3%), kuid usaldusväärselt vähem kui Le(b–) fenotüübiga doonoritel (χ^2 = 7,31; p = 0,006).

Sekretoorne staatus (*Se/se*) (vt tabel 3)
Mao healoomuliste haigustega patsientide rühmas ei mõjutanud sekretoorne staatus oluliselt *H. pylori* infektsiooni esinemissagedust: erinevused sekreetorite ja mittesekreetorite gruppides ei olnud statistiliselt usaldusväärsed ning moodustasid 3–7% erinevates uuringurühmades. CagA-positiivsete isikute protsent sekreetorite ja mittesekreetorite hulgas oluliselt ei erinenud (vastavalt 79,6% ja 73,9%). Kaksteistsõrmiksoole-haavandiga haigete seas oli aga usaldusväärselt suurem mittesekreetorite protsent (45st 15; 33,3%) võrreldes kroonilise gastriidiga haigetega (kõik gastriidi vormid, ühendgrupp) (χ^2 = 6,9; p = 0,0089). Teistes gruppides oli see näitaja 9,5–12,5%. Kaksteistsõrmiksoole-haavandiga haigete seas oli CagA-positiivsete isikute protsent suurem mittesekreetorite seas (15st 12; 80%) võrreldes teiste haigete mittesekreetorite gruppidega (50–66,6%), kuid erinevused ei olnud statistiliselt usaldusväärsed.

Arutelu
Me ei leidnud olulisi erinevusi *H. pylori* infektsiooni esinemissageduses mao erinevate haiguste korral. Arvata võib, et tingimustes, kus absoluutne enamus populatsioonist on *H. pylori* infektsiooni nakatunud, ei saagi oodata suuri erinevusi. Öeldu puudutab ka CagA staatust, kuigi maohaavandihaigete seas oli märgatavalt suurem CagA+ fenotüübi esinemissagedus. Analoogne situatsioon on iseloomulik ka teistele *H. pylori*'ga nakatunud ja CagA-seropositiivsete isikute suure esinemissagedusega regioonidele (9–11).

CagA fenotüpeerimine immunoblottingu meetodil näitas CagA-positiivsete isikute usaldusväärselt väiksemat esinemissagedust A-veregrupiga veredoonorite seas (p = 0,01). Sama tulemuse saime ka mao healoomuliste haigustega patsientide uurimisrühmas, mis räägib A-veregrupiga inimeste suuremast resistentsusest *H. pylori* CagA-positiivsete tüvede suhtes. Analoogsed andmed saadi ka varem immunoensüümmeetodil, kus antigeenina kasutati CagA valgu rekombinantset fragmenti (16). Sellise seose puudumist maovähihaigete grupis võib arvatavasti põhjendada A-veregrupiga isikute väikese arvuga selles uuringurühmas (23,2%; 138st 32) võrreldes doonorite (35%; 144st 44; p = 0,1) ja mao

healoomuliste haigustega patsientidega (32,6%; 98st 32; p = 0,07).

Leidsime, et Le(a+) fenotüübiga isikute seas on rohkem *H. pylori* CagA+ fenotüübiga nakatunuid. Varem näitasime, et mittesekreetorid nakatuvad *H. pylori* infektsiooni harvemini ning *H. pylori*'le suunatud IgG immuunvastus on nendel nõrgem (5). Neid andmeid interpreteerisime nii, et mittesekreetorid on resistentsemad selle infektsiooni suhtes, kuna nendel puudub Le(b) antigeen, mis on *H. pylori* Le(b)-spetsiifilise BabA adhesiini ligandiks (18). Virulentsemate CagA+ *H. pylori* tüvede ülekaal selles rühmas on kaudseks tõendiks meie ideele, kuna oleks loogiline oodata, et resistentsem indiviid võib nakatuda ainult palju virulentsema mikroobitüvega. Seega "määrab" peremeesorganism ise, millise mikroobitüvega nakatuda. ABH ja Lewisi fenotüüpide leviku populatsioonilised ja geograafilised variatsioonid võivad osaliselt seletada erinevusi mitmete *H. pylori* infektsiooniga seotud haiguste levikus.

Segainfektsioonide (CagA-negatiivsete ja -positiivsete tüvede esinemine ühel ajal maos) suur sagedus (12) võib osutuda tõsiselt segavaks teguriks CagA staatuse testimisel, kui kasutatakse DNA-l rajanevaid meetodeid. Seoses sellega oletame, et seroloogilise meetodi kasutamine peegeldab situatsiooni palju adekvaatsemalt, sest lubab määrata domineeriva CagA fenotüübi.

Lewisi fenotüübi seos peremehe immuunreaktsiooni tasemega (5, 14) näitab, et mukoosa rakkude glükokonjugaatide ja mutsiinide struktuur võib oluliselt mõjutada peremehe ning mikroobi vahekorda. Sama kehtib ka mitmete teiste mikroobide kohta, kellel on retseptorid vere grupiantigeenide (ABH, Lewis) ning nende derivaatide vastu (13). Erinevalt Lewisi fenotüübist ei mõjuta sekretoorne staatus oluliselt *H. pylori* CagA-positiivsete tüvedega nakatumist. Nähtavasti on palju tähtsamaks teguriks Le(a,b) antigeenide ekspressioon mao limaskesta rakkude glükokonjugaatide pinnal ja mitte ABH ning Lewisi antigeenide sekretsioon maomahla. Nii *H. pylori* kui ka CagA suur esinemissagedus (81,4 ja 83,1%) erinevate healoomuliste haigustega patsientidel lubab oletada, et peale Lewisi fenotüübi ja sekretoorse staatuse on ka teistel teguritel tähtis roll selle infektsiooni kliinilise tagajärje määramisel.

Üha rohkem andmeid koguneb selle kohta, et peremeesorganism määrab mitte ainult anti-*H. pylori* immuunreaktsioonide omadused (lokaalsete ja süsteemsete immuunreaktsioonide tugevus, rakuliste ja humoraalsete immuunreaktsioonide ning ka põletiku poolt ja vastu tsütokiinide vahekord), vaid olulisel määral ka *H. pylori* enda omadused, sh virulentsustegurite ekspressiooni (5, 8, 14, 15, 16, 19, 20). Kõik see kokku määrab gastriidi aktiivsuse, *H. pylori* kolonisatsiooni tiheduse mukoosas, põletiku aktiivsuse, mao limaskesta atroofia arenemise kiiruse ning selle tagajärjed, sh maovähi tekkimise. See käib eriti nn aeglaste infektsioonide (nagu *H. pylori* infektsioon) kohta, kus mikroobi ja peremeesorganismi vahekord kestab aastaid ning toob kaasa maksimaalse vastastikuse adaptatsiooni.

Kokkuvõte

Eesti kuulub suure *H. pylori* infektsiooni nakatumise ja *H. pylori* CagA-positiivsete (virulentsemate) tüvede suure levikuga regioonide hulka. Peremeesorganismi ABO(H) ja Lewis(a,b) fenotüüp mõjutavad oluliselt CagA seroloogilist staatust: 1) A-veregrupiga doonorite ja mao healoomuliste haigustega isikute seas on usaldusväärselt väiksem CagA-positiivsete tüvede levik; 2) Le(b-) fenotüübiga doonorid nakatuvad sagedamini *H. pylori* CagA-positiivsete tüvedega kui teiste Lewisi fenotüüpidega isikud. Maovähi hilisemates staadiumides haigete seas on vähem *H. pylori* ja CagA-positiivseid isikuid. Need seosed on üldise iseloomuga ning sõltuvad vähe haigusest. Võrdlevatel epidemioloogilistel uuringutel, mille eesmärgiks oli selgitada *H. pylori* infektsiooni rolli mao ja kaksteistsõrmiksoole krooniliste haiguste patogeneesis, on vajalik arvestada ABO(H) ning Lewisi fenotüüpide jaotumist uuritavas populatsioonis.

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Summary

***Helicobacter pylori* and CagA serologic status in patients with gastroduodenal pathology: relation to ABO(H), Lewis (a,b) phenotype and Se/se status of the host**

The seroprevalence of *H. pylori* and the CagA positive strain in blood donors and in patients with gastroduodenal pathology was tested in relation to ABO(H), Lewis(a,b) phenotype and the secretory (*Se/se*) status of the host. High *H. pylori* seroprevalence (74–86%) was found in all studied groups including blood donors (79.1%). Compared with blood donors, significantly higher CagA seroprevalence was revealed in patients with gastric cancer and peptic ulcer disease. A decrease in the prevalence of *H. pylori* and CagA was noted in patients with advanced cancer. Donors of blood group A and those with peptic ulcer disease showed a lower CagA seropostivity rate ($p<0.05$) compared with the related groups of the other phenotypes. Donors of the Le(b-) phenotype were significantly more often infected with CagA positive strains. The proportion of non-secretors was significantly higher among patients with duodenal ulcer. However, the secretory status was not related to the CagA status. The data suggest that ABH and the Lewis phenotype of the host may significantly influence the susceptibility of the host to be infected with a particular genotype of *H. pylori*. This should be taken into consideration in epidemiological and immunological studies of *H. pylori*-host interplay.

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VI

Klaamas K, Kurtenkov O, Brjalin V, Miljukhina L, Shljapnikova L, Engstrand L. Enhanced humoral immune response to tumor-associated T glycotope (Galβ1,3-GalNAc) in *Helicobacter pylori*-infected blood donors, patients with gastric cancer and benign gastric conditions. Exp Oncol 2002; 24: 38–44.

ENHANCED HUMORAL IMMUNE RESPONSE TO TUMOR-ASSOCIATED T GLYCOTOPE (GALβ1,3–GalNAc) IN *HELICOBACTER PYLORI*–INFECTED PATIENTS WITH GASTRIC CANCER AND NON-TUMOR GASTRIC DISEASES

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УСИЛЕНИЕ ГУМОРАЛЬНОГО ИММУННОГО ОТВЕТА НА ОПУХОЛЕАССОЦИИРОВАННЫЙ Т-ГЛИКОТОП (GALβ1,3–GalNAc) У ПАЦИЕНТОВ СО ЗЛОКАЧЕСТВЕННЫМИ И НЕОПУХОЛЕВЫМИ ЗАБОЛЕВАНИЯМИ ЖЕЛУДКА, ИНФИЦИРОВАННЫХ *HELICOBACTER PYLORI*

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Natural carbohydrate-binding antibodies are considered to play a role in host defence against infections and neoplasia. We aimed to study whether *H.pylori* infection influences natural immune response to tumor-related T glycotope. A level of T-specific antibodies in serum of *H.pylori*-infected and uninfected blood donors (n = 191), patients with gastric cancer (n = 186) and non-tumor gastric disorders (n = 126) was detected by ELISA using T-disaccharide-polyacrylamide conjugate as antigen. *H.pylori* status was evaluated with ELISA and Lewis (a, b) phenotyping of erythrocytes with monoclonal antibodies. The Se/se status was determined by testing of H-antigen in saliva. Expression of T epitope in *H.pylori* was evaluated by immunoblotting. An increased IgG response to T antigen was observed in all *H.pylori*-infected groups compared to uninfected controls. The differences were significant for patients with gastric cancer and gastritis ($p < 0.05$). The *H.pylori*-uninfected patients with peptic ulcer revealed the lowest level of IgG T-antibody. An increased IgG response in infected individuals was mostly related to the subjects of Le(b-) phenotype. A decrease of T antibody level was observed after *H.pylori* eradication in patients with high level of antibodies before treatment. In *H.pylori* extracts, two protein bands (58 kDa and 68 kDa) were immunostained with T epitope-specific monoclonal antibodies. In conclusion, the *H.pylori* infection is associated with an increased immune response to cancer-associated T epitope. This systemic impact appears to be dependent on Lewis phenotype of the host, in part disease type-specific and might be explained by T epitope expression in *H.pylori*. These findings suggest that *H.pylori* may be indirectly involved in gastric carcinogenesis *via* modulation of natural cancer-related immune mechanisms.
***Key Words*: *Helicobacter pylori*, Thomsen – Friedenreich antigen, host factors, tumor-associated antigens, gastric cancer, peptic ulcer.**

Естественные антитела к углеводным эпитопам участвуют в защите организма против инфекций и опухолевого роста. Мы исследовали, влияет ли инфекция *H.pylori* на уровень естественных антител к опухолеассоциированному Т-антигену у людей разного Lewis-фенотипа и *Se/se*-статуса. Уровень Т-эпитопспецифичных антител в крови у *H.pylori*-инфицированных и неинфицированных доноров (191), больных раком (186) и пациентов с неопухолевыми заболеваниями (126) желудка определяли с помощью иммуноферментного метода с использованием в качестве антигена Т-дисахарид-полиакриламидного конъюгата. Серологический статус *H.pylori* определяли иммуноферментным методом; Lewis(a,b)-фенотип – с помощью моноклональных антител; Se/se-статус – по уровню Н-антигена в слюне. Экспрессию Т-эпитопа *H.pylori* оценивали с помощью метода иммуноблоттинга. Повышенный уровень IgG Т-антител наблюдали во всех *H.pylori*-инфицированных группах по сравнению с неинфицированными; различия были достоверны для больных раком желудка и гастритом. Это повышение было более выражено у лиц Lewis(b)-фенотипа. У больных с пептической язвой отмечены самые низкие уровни IgG Т-антител. В экстрактах мембран *H.pylori* выявлены два белка (58 кД и 68 кД), связывающие Т-антигенспецифические моноклональные антитела. Излечение инфекции *H.pylori* приводило к существенному снижению исходно высокого уровня Т-антител. Таким образом, инфекция *H.pylori* ассоциирована с повышением уровня естественных антител к опухолеассоциированному Т-гликотопу. Это системное влияние инфекции обнаруживает связь с Le-фенотипом хозяина, что отчасти специфично для определенных заболеваний и, возможно, связано с экспрессией Т-эпитопа на *H.pylori*. По данным исследования, предполагается участие *H.pylori* в канцерогенезе путем модуляции естественных противоопухолевых механизмов.
***Ключевые слова*: *Helicobacter pylori*, антиген Томсена – Фриденрейха, факторы хозяина, опухолеассоциированные антигены, рак желудка, пептическая язва.**

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Natural immune response to tumor-related carbohydrate epitopes is considered to be one of the natural defence mechanisms against cancer [1–4]. The Thomsen — Friedenreich (T) antigen (Galβ1,3-GalNAcα/β-O-Ser/Thr) is a blood-group related tumor-associated molecule which is expressed in a majority of carcinomas and related to invasion, metastasis and prognosis [5, 6]. Natural antibodies to T epitope (T antibodies) are present in every individual. Their synthesis was considered to be dependent on the antigenic stimuli derived from T antigen-positive intestinal flora [5]. A level of T antibodies is fairly stable for a given individual but there are appreciable and unexplained interindividual variations. A dramatic decrease in serum T antibody level has been observed in patients with cancer [5–9]. It has been also shown that low level of T-specific IgM antibodies is associated with an increased risk for cancer [5, 7].

We have reported earlier that the individuals suffering from the non-tumor gastric diseases also showed very low level of IgM T antibodies [9]. This suggests that anti-T humoral immune response may be altered by factors unrelated to cancer but associated with non-tumor gastric diseases. The *H.pylori* infection is very closely related to gastroduodenal pathology [10, 11] and might be one of the operating factors which alter immune response to T epitope.

The blood-group related carbohydrate antigens such as ABH, H-1, Lewis type 1 and type 2 are expressed in *H.pylori* [12–15]. Their expression was shown to be associated with host inflammatory response to *H.pylori* [12–16]. We found recently that serum level of natural IgM antibody to tumor-related T epitope was dependent on *H.pylori* serologic status [17] and related to the Le phenotype of the host [8]. Similar associations were shown for anti-Lewis type 2 IgG antibodies [18].

We hypothesized that *H.pylori* infection may alter immune response to tumor-related carbohydrate epitopes thus modulating natural resistance against cancer and host — *H.pylori* relationships. The aim of this study was to investigate whether the *H.pylori* infection has any impact on IgM and IgG immune response to tumor-associated T glycotope in blood donors, patients with gastric cancer and non-tumor gastric diseases. In addition, a possible link to Lewis (a, b) phenotype of the host, as well as an effect of the *H.pylori* eradication were explored. We report here that the *H.pylori* infection is associated with an increased IgG immune response to T epitope.This impact appears to be host-dependent and in part disease type-specific. We also present an evidence that T epitope is expressed in *H.pylori*.

MATERIALS AND METHODS

Subjects and serum samples. Alltogether 503 individuals before any treatment were investigated (Table 1). The patients with histologically verified gastric carcinoma were classified according to the histopathological TNM classification. All patients with non-tumor gastric diseases underwent upper gastrointestinal endoscopy. Biopsy specimens were taken from the antral and corpus mucosa and analysed by Sydney system. The sera from patients and randomly selected blood transfusion donors were separated after blood clotting and stored at –20 °C until studied.

Antigen preparation for H.pylori serology. A glycine cell surface antigen extraction of *H.pylori* strain NCTC 11637 was performed according to [19]. The strain was grown on *Gonococcal* agar base/*Campylobacter* reference agar at 37 °C for 2 days in plastic jars filled with a gas mixture of 5% O_2, 10% CO_2 and 85% N_2. A pool of nine different preparations was used as antigen in *H.pylori* ELISA and immunoblotting.

H.pylori enzyme-linked immunosorbent assay (HP-ELISA). Serum samples were examined by ELISA as described earlier [20, 21]. In brief, 96-well flat bottom microtitre plates (Maxi Sorp, Nunc, Roskilde, Denmark) were coated with 0.1 ml of antigen (5 μg/ml) per well and incubated at 4 °C overnight. Alkaline phosphatase conjugated goat anti-human IgG (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) and p-nitro-phenyl-phosphate (Sigma, St. Louis, MO, USA) as substrate were used. A pool of human IgG (Kabi AB, Stockholm, Sweden) was placed in each ELISA plate as a positive control for 100 units as well as *H.pylori* seronegative reference serum was run for negative control. The relative antibody activity (RAA) values were calculated according to [22]. RAA values ≤ 25 were regarded as negative. A specificity of the test has been reported of 97.4% and a sensitivity — 89.7% [23]. The subjects with "grey zone" RAA values (26–40) were not included in this study.

T-epitope-related proteins in cell surface membrane extract of H.pylori. Immunoblotting was performed as described earlier [24]. The membranes (Immobilon P, Millipore, Bedford, MA, USA) were treated with *H.pylori* seropositive human serum or with T epitope-specific monoclononal antibodies (MAb). Human MAb TF1, (IgM), was kindly provided by Dr. B. Jansson (BioInvent Therapeutics AB, Lund, Sweden) and murine MAb 3C9 (IgM) from Dr. H. Clausen (Copenhagen, Denmark). Unrelated murine monoclonal antibody 19 — OLE (IgM) specific for H type 2 antigen was

Table 1. Characteristics of the subjects and examined parameters

Group	n	Males/females (ratio)	Median age, (range)	*H. pylori*-positive (%)	T-specific antibody		Lewis type or Se/se status
					IgM	IgG	
Blood donors	191	105/86 (1.22)	49.2 (26–65)	145 (75.9)	191	107	191
Patients with gastric carcinoma	186	119/67 (1.78)	59.9 (28–79)	134 (72.0)	186	177	77
Non-tumor gastric diseases:	126	78/48 (1.62)	49.8 (31–76)	101 (80.1)	126	122	84***
peptic ulcer disease*	72	47/25 (1.88)	41.3 (27–52)	58 (80.5)	72	72	45
chronic gastritis**	54	31/23 (1.34)	51.7 (39–76)	43 (79.6)	54	50	39
Total	503				503	406	352

* Peptic ulcer disease: duodenal ulcer — 53, stomach ulcer — 19.

** Atrophic gastritis — 31 patients, nonatrophic gastritis— 23. The patients with a moderate or severe atrophy in any part of the stomach as evaluated by Sydney system were considered as having an atrophic gastritis.

*** These patients with non-tumor gastric diseases were tested for secretor/nonsecretor status (*see Methods*). Secretors — 68, nonsecretors — 16.

a gift from Dr. J. Bara (Villejuif, France). The specifity of Mab's was characterized elsewhere [25–27]. The strips were incubated with Mab's overnight at 4°C in PBS — 0.5% Tween–20, washed 3 times with PBS — 0.05% Tween–20 followed by incubation with alkaline phosphatase–labelled rabbit anti–human IgM (1 : 2000) (Dako A/S, Glostrup, Denmark), or rabbit anti–mouse immunoglobulins–alkaline phosphatase (Dako) and developed with NBT/BCIP substrate (Sigma, St.Louis, MO, USA).

Lewis (a, b) phenotyping and secretor/nonsecretor status evaluation. Lewis phenotyping of erythrocytes was carried out using anti–Le^a and –Le^b monoclonal antibody gel system (DiaMed, Switzerland) according to the instruction of the manufacturer. In patients with non–tumor gastric diseases the Se/se status was determined by testing of H–antigen in boiled saliva at dilution 1 : 10 using H–specific biotin–labelled lectin UEA–1 from *Ulex europaeus* (Sigma) as described by [28]. The method was compared with Le erythocyte testing in 25 individuals and the results were identical in 96% of cases among the subjects of Le(a+b–) and Le(a–b+) phenotype. All non–secretors showed OD values below 0.25. No overlapping for OD values was observed in both groups except 1 donor of Le(a–b+) phenotype. He was classified as Le(a–b+)/secretor as detected with MAb but defined as nonsecretor by UEA–I lectin assay. Apparently he belonged to the so–called "weak" secretors [29].

Determination of serum anti–T IgM and IgG antibodies by ELISA. These assays were carried out as described earlier [8]. Plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with synthetic T–hapten–polyacrylamide (PAA) conjugate (Synthesom, Munich, Germany; 10 mol% of carbohydrates) 2 μg/ml in carbonate buffer 50 mM Na_2CO_3/$NaHCO_3$ and 0.02% NaN_3, pH 9.6 or 1% BSA in PBS (control wells) at 4 °C overnight. After washing three times with PBS–Tween (0.13 M NaCl, 8 mM NaH_2PO_4/KH_2PO_4, 3 mM KCl, 0.02% sodium azide, pH 7.2, 0.05% Tween 20) the plates were blocked with 0.15 ml of 1% BSA in PBS for 1 h at room temperature (RT) and washed in PBS–Tween. Serum (100 μl) diluted 1 : 500 in PBS–Tween was added and incubated overnight at RT. The plates were then washed and bound IgM was detected with 100 μl of alkaline phosphatase conjugated rabbit anti–human IgM (Sigma, St.Louis, MO) diluted 1 : 500 in PBS–Tween. Following an incubation for 90 min at RT and washing, the plates were developed with *p*–nitro–phenyl–phosphate (Sigma), 1 μg/ml in 1 M diethanolamine buffer, pH 9.8 for 30 min and absorbance values at 405 nm were registered with Labsystem Multiscan MCC/340 (Finland). Anti–T IgG antibodies were determined by a similar procedure except the plates were precoated with T hapten–PAA conjugate at 5 μg/ml, the serum diluted 1 : 50 was incubated for 2 h at RT, and alkaline phosphatase conjugated goat anti–human IgG (Gibco, BRL, Life Technologies, Gaithersburg, MD, USA) 1 : 1000 was used. An optical density (O.D.) values of control wells (PBS–BSA) was subtracted from the values of the wells coated with the T conjugate and net O.D. values more than 0.4 and 0.3 were considered as strong IgM and IgG response, respectively. These cut–off limits were calculated as described elsewhere [8]. Each serum was analysed in duplicate. Two reference serum from weak and strong responders were run in every plate as internal standards to control the experimental conditions. The intraassay variations did not exceed 7%.

H.pylori eradication therapy. Eight patients with duodenal ulcer were treated with a standard one week triple therapy (amoxycillin 1.0 g, clarithromycin 0.5 g, and omeprasol 20 mg twice a day) and seven untreated control subjects were follow–up'ed for 6–24 month. Serum samples taken before and at different intervals (6–12 months) after therapy were stored at –20 °C and tested in parallel. The decrease of IgG *H.pylori* antibody level (RAA) at 6 month after therapy more than by 50% was considered as a sign of successful treatment.

Statistical analysis. Statistical comparisons between the groups were performed by Fisher's exact test. Odds ratios (ORs) and their 95% confidence intervals (95% CI) were calculated to measure an association between the parameters studied. The difference was considered to be significant when $p < 0.05$.

RESULTS

The proportions of *H.pylori*–seropositive and –negative individuals did not appreciably differ between the groups studied (72–80.5%). The changes in IgM and IgG T–antibody level in relation to the *H.pylori* serologic status of patients and blood donors are shown in Fig. 1.

IgM antibody: In blood donors, 58.7% and 76.5% of individuals were strong IgM responders for *H.pylori*–negative and –positive subjects, respectively: $p = 0.02$; OR — 2.3; 95% CI — 1.1–4.9). The proportion of strong IgM responders was not appreciably influenced by Lewis phenotype except the individuals of Le(a–b+)/secretor phenotype who showed a significantly higher response for *H.pylori*–seropositive subjects compared to seronegative subgroup ($p = 0.004$) (Fig. 2A). No significant difference was observed between the *H.pylori*–seronegative subgroups of various Lewis phenotype.

As compared with blood donors, a highly significant decrease in the proportion of strong IgM responders was observed in cancer patients as well as in the patients with non–tumor gastric diseases irrespective of *H.pylori* serology or disease stage (see Fig. 1; Table 2). Unlike blood donors, a higher proportion of strong responders in *H.pylori*–seropositive group was observed among cancer patients of Le(b–) phenotype ($p = 0.038$) compared with the Le(b+) patients (Fig. 2B).

In the group of patients with non–tumor diseases, the secretors showed the patterns similar to those in cancer patients of Le(a–b+) phenotype, i.e. very low IgM response irrespective of *H.pylori* status. The non–secretors (*H.pylori*–positive — 10 and –negative — 6) revealed no strong responders at all. The patients with stomach and duodenal ulcer showed similar proportions.

Thus both the patients with gastric cancer and non–tumor gastric diseases revealed very low IgM response to T epitope compared to blood donors. These differences were not related to *H.pylori* status but in part influenced by Lewis phenotype of the host.

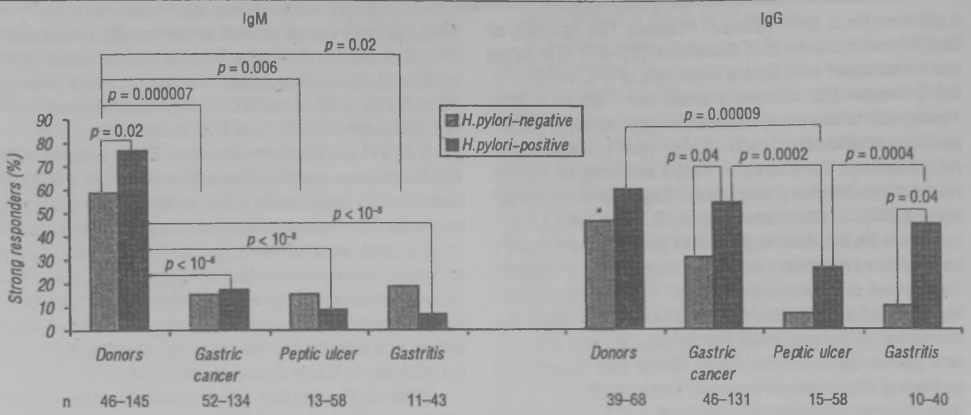

Fig. 1. The proportion of IgM and IgG strong responders to T epitope in blood donors and patients by *H.pylori* serology. *P* values are shown for significant differences as calculated by Fisher's exact test.
* Significantly higher compared with *H.pylori*–seronegative group of patients with "peptic ulcer" ($p = 0.005$) and "gastritis" ($p = 0.04$); n — the number of individuals for *H.pylori*–seronegative and *H.pylori*–seropositive groups respectively.

Table 2. The proportion of strong responders to T-epitope in patients with gastric carcinoma by stage of the disease

T antibody isotype	*H. pylori* status	Stage of the disease*				All stages
		I	II	III	IV	
IgM	—	0 (8)**	10.0 (10)	14.3 (21)	30.8 (13)	15.4 (52)
	+	13.6 (22)	21.7 (23)	20.5 (39)	14.0 (50)	17.2 (134)
IgG	—	42.8 (7)	33.3 (9)	22.2 (18)	33.3 (12)	30.4 (46)
	+	59.1 (22)	56.5 (23)	64.1*** (39)	42.5 (47)	54.2*** (131)

* Based on the international TNM classification.
** The proportion (%) of strong responders; in brackets – the number of patients.
*** A significantly higher proportion of strong responders compared with the related H. pylori–seronegative groups.

IgG antibody: The main changes related to the *H.pylori* status were noted for IgG antibody level in the patients with gastric cancer and non–tumor gastric diseases (see Fig. 1). The *H.pylori*–seropositive patients with non–tumor gastric disorders (combined group) showed a significantly higher proportion of strong IgG responders (34%) than related seronegative group (8%, $p = 0.007$). The same was true for the patients with gastric cancer: 54.2% and 30.4% for *H.pylori*–infected and uninfected patients, respectively; $p = 0.004$. It is to note that the *H.pylori*–seropositive group of cancer patients did not differ significantly from the related group of blood donors ($p = 0.25$) whereas the *H.pylori* seropositive non–tumor group showed a significantly lower proportion of strong responders compared with both blood donors and cancer patients ($p = 0.0007$ and 0.002 respectively). Among the *H.pylori*–seropositive individuals the lowest level of IgG T–antibody was found in patients with peptic ulcer disease (25.8% of strong responders). This is a significantly lower than in related group of cancer patients (54.2%, $p = 0.002$) and blood donors (60.3%; $p = 0.00009$; OR — 4.4; 95%CI 1.9 — 10.1. The *H.pylori* seropositive patients with chronic gastritis did not reveal such difference from donors and cancer patients. In the non–tumor group, both the *H.pylori*–infected secretors and nonsecretors showed an insignificantly higher proportion of strong IgG responders than related *H.pylori*–negative subgroups: 37.3% and 30% for *H.pylori* seropositive secretors and nonsecretors versus 12.5% and 0% for *H.pylori*–seronegative secretors and nonsecretors, respectively. This difference was mostly related to *H.pylori*–infected patients with chronic gastritis: 12 of 25 secretors (48%) and 2 of 4 (50%) non–secretors were strong IgG responders. The seronegative subgroups were too small for comparison (peptic ulcer — 7, chronic gastritis — 7).

Unlike IgM response, a significantly higher proportion of strong IgG responders was found in *H.pylori*–seropositive donors of Le(b–) type (see Fig. 2B) compared with the related seronegative group ($p = 0.04$). The *H.pylori*–infected Le(b+)/secretors showed an increase in IgM T antibody level whereas IgG response was increased in Le(b–) group of the infected subjects. The patients with cancer showed a similar trend but the difference did not reach the statistical significance possibly due to a small size of the *H.pylori*–seronegative and Le(b–) subgroups (see Fig. 2B).

The proportion of strong responders did not differ significantly between the patients with atrophic and non–atrophic gastritis or duodenal versus stomach ulcer. Similarly, no significant difference for IgM antibody level was noted for the patients at different stages of cancer (see Table 2). However, the proportion of IgG strong responders was appreciably higher in *H.pylori*–seropositive cancer patiens at all stages of the disease. The difference was significant for the patients at stage III and the combined group.

Immunodetection of T epitope–related proteins of H.pylori by immunoblotting. On blots, both human (TF1) and murine (3C9) T–specific monoclonal antibody but not H type 2 antigen–specific control Mab (19OLE) immunostained two protein bands with molecular weight ~58 kDa and ~68 kDa (Fig. 3).

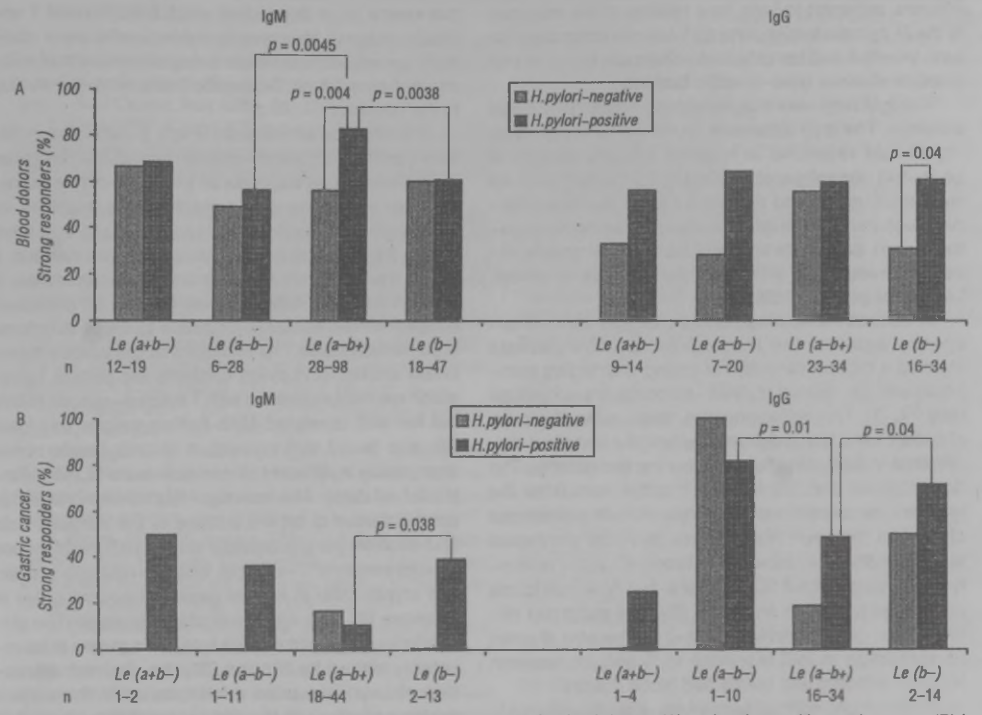

Fig. 2. The proportions of IgM and IgG strong responders to T epitope in blood donors (A) and patients with gastric cancer (B) in relation to *H.pylori* status and Lewis(a,b) phenotype. *P* values are shown for significant differences as calculated by Fisher's exact test. Le(b–) combined group: Le(a+b–) plus Le(a–b–) individuals

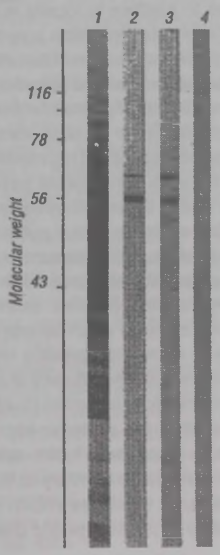

Fig. 3. T epitope–related protein bands on immunoblots of NCTC 11637 *H.pylori* strain
The strips were immunostained with: *lane 1*: *H.pylori* seropositive serum (IgG antibody profile); *lane 2*: human monoclonal antibody TF1; *lane 3*: murine monoclonal antibody 3C9; *lane 4*: murine anti–H type 2 monoclonal antibody 19OLE (unrelated IgM isotypic control)

Effect of H.pylori eradication. Only 3 of 8 treated patients were strong IgM responders before therapy. Two of them were successfully treated and showed an appreciable (70% and 500%) decrease of IgM T–antibody level (OD values). The only IgG strong responder revealed a 170% decrease after successful treatment. Three patients with peptic ulcer who were treated unsuccessfully and remained strongly *H.pylori*–seropositive for 6–12 months after treatment did not reveale appreciable changes in T antibody level and one strong IgG responder showed further increase of IgG antibodies. Seven untreated individuals (2 — *H.pylori* seronegative and 5 — seropositive) were tested repeatedly (2–4 times) for IgM and IgG T–antibody level in serum during 6–24 months. Six of them revealed no appreciable changes: the OD values did no vary more than by ± 26%. One subject showed a higher IgM response with no difference in IgG level. All treated patients were the secretors.

The data show that the successful treatment of the *H.pylori* infection may decrease a level of T–antibody in some patients. The failed therapy did not alter T antibody level.

DISCUSSION

The changes in T antibody levels were analysed in two ways: by comparison (i) between the *H.pylori*–seronegative and –positive subgroups within each group

(donors, patients) to look for a relation of the changes to the *H.pylori* infection, and (ii) between the groups for both infected and noninfected individuals trying to find putative disease type–specific features.

The IgM response was not closely related to *H.pylori* serology. The only difference found was a significantly higher IgM response in *H.pylori* Infected donors of Le (a–b+) phenotype compared to uninfected ones. As compared with blood donors a highly significant decrease in the level of IgM T antibody was found in patients with gastric cancer and non–tumor gastric diseases irrespective of *H.pylori* status, stage of cancer, Le type or secretory status.

In contrast, a common feature for IgG immune response was that the *H.pylori*–seropositive patients showed a higher proportion of strong responders compared with the related *H.pylori*–seronegative subgroups (see Fig. 1). This difference was observed in all groups of patients and blood donors reaching the statistical significance in patients with gastric cancer and gastritis. The data suggest that the *H.pylori* infection stimulates the IgG immune response to T–epitope. A more pronounced difference between *H.pylori*–infected and uninfected blood donors was observed in those of Le(b–) phenotype. Although about 90% of Le(a–b–) type individuals are known to be the secretors [29] this subgroup differed from the secretors of Le(a–b+) type who showed no distinction in IgG response to T antigen between *H.pylori*–infected and uninfected blood donors.

Interestingly, some disease type–specific patterns in T response may also be seen. The *H.pylori*–seropositive patients with gastric cancer and gastritis showed the IgG response similar to that of donors. At the same time, the *H.pylori*–seropositive patients with peptic ulcer disease revealed only slight increase in IgG response: a proportion of strong responders was significantly lower than in any other *H.pylori*–seropositive group.

It remains unclear whether a lower level of T–antibody in *H.pylori*–seronegative patients with cancer and chronic gastric diseases is due to the disease *per se* or these individuals have been weak responders prior to disease development. We did not find any relation of T antibody level to the stage of cancer. In fact, IgM and IgG T antibody level in uninfected patients was not dependent on the stage of cancer. In contrast, a higher proportion of IgG strong responders was mostly found in *H.pylori*–infected cancer patients irrespective of the stage of cancer. This suggests that the changes observed should not be considered as a secondary tumor–induced event. Since a level of natural antibodies is reported to be fairly stable for years [2, 5] we suggest that there is some kind of enrichment of weak T responders among the individuals which are predisposed to gastric pathology or suffering from chronic gastric diseases including cancer. An association of T–antibody level with Le(a,b) histo–blood group phenotype suggests a possible genetic background.

Our preliminary data about the decrease of immune response to T epitope in some patients after *H.pylori* eradication further support the idea that an increase in T antibody level is related to *H.pylori* infection. The effect seems to be dependent on an initial level of T antibody and was observed in subjects who were obviously low responders before being infected with *H.pylori* and enhanced their T–specific immune response due to the infection.

A similarity in an increase of IgG T–response in different groups of *H.pylori*–infected individuals compared to uninfected ones suggests an existence of some common pathway. One of the mechanisms might be an expression of T epitope in *H.pylori*, like it has been shown for many carbohydrate epitopes on bacteria. If this is the case, an ongoing antigenic stimulation in *H.pylori*–infected subjects may lead to an increased antigen–driven antibody response to these structures in some individuals. Our findings that cell surface membrane extract of *H.pylori* contains the protein bands which are immunostained with T epitope–specific MAb's but not with unrelated MAb further support this idea. We also found that proportion of both bands varied appreciably in different clinical isolates of *H.pylori* (unpublished data). Alternatively, *H.pylori* who lives within gastric mucus or on the surface of the mucosal cells and displays the glycosidase activity [30] might induce an expression of T–epitope, which is normally present in a cryptic form in normal gastric glycoconjugates of secretors [31, 32], with the similar sequealae. The glycosylation of gastric mucins has been shown to be reversibly altered by *H.pylori* [33]. An aberrant expression of Le(x) has been demonstrated in the surface gastric epithelium of *H.pylori*–infected individuals [34]. This is in accordance with our findings about the enhanced IgG response to Le(x) in infected subjects [18]. We suggest that, in addition to locally *in situ* operating mechanisms, the *H.pylori* infection may be involved in gastric pathology *via* modulation of natural immune response to tumor–associated carbohydrate epitopes.

We can conclude that *H.pylori* infection is associated with a significantly higher humoral immune response to tumor–associated T epitope. This impact was strongly pronounced in *H.pylori*–seropositive patients with any stage of cancer and in those with chronic gastritis. Our findings is the first evidence that *H.pylori* infection may appreciably modulate humoral immune response to tumor–related T epitope. We also present an evidence that T–epitope is expressed in *H.pylori*. Both humoral and cell–mediated immunity to such simple carbohydrate epitopes has been shown to correlate with the clinical course of cancer and with the efficacy of cancer immunotherapy with mucin–type vaccines [3–6]. It remains to be elucidated, whether the systemic impact of *H.pylori* infection on immune response to tumor–related T epitope may work in concert with or contrary to the other *in situ* operating mechanisms which are known to be involved in the pathogenesis of chronic gastric diseases including cancer.

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VII

Klaamas K, Kurtenkov O, Rittenhouse-Olson K, Brjalin V, Miljukhina L, Shljapnikova L & Engstrand L. Expression of tumor-associated Thomsen-Friedenreich antigen (T Ag) in *Helicobacter pylori* and modulation of T Ag specific immune response in infected individuals. Immunol Investigations 2002; 31: 191–204.

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EXPRESSION OF TUMOR-ASSOCIATED THOMSEN-FRIEDENREICH ANTIGEN (T Ag) IN *HELICOBACTER PYLORI* AND MODULATION OF T Ag SPECIFIC IMMUNE RESPONSE IN INFECTED INDIVIDUALS

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ABSTRACT

We tested the hypothesis that the gastric cancer associated bacteria, *Helicobacter pylori* (*H. pylori*) express the cancer-related Thomsen-Friedenreich (T) antigen. We also analysed whether infection with *H. pylori* alters the amount of natural anti-T antibodies in the patients' sera. Cell surface membrane extracts of *H. pylori* NCTC 11637 strain and clinical isolates of *H. pylori* (n = 13) were analysed by immunoblotting and cell-ELISA with five different T antigen-specific monoclonal antibodies (MAbs). Two major protein bands of ~68 kDa and 58 kDa were immunostained on blots of *H. pylori* extracts with T specific MAbs but not immunostained with unrelated MAb. The specificity was shown

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in that immunostaining was blocked with peanut agglutinin (PNA) and rabbit antiserum to T antigen. The binding of T specific MAb to the 58 kDa protein band was also blocked by rabbit antiserum against heat shock proteins of *H. pylori*. The relative expression of T antigen-related proteins differed among *H. pylori* strains, with 68 kD associated T antigen expression higher in patients with more severe pathology. The level of IgG antibody to T epitope in patients with gastric cancer ($n = 66$) and normal blood donors ($n = 62$) were compared and the level of anti-T Ab in gastric cancer patients was significantly lower than that in normal blood donors. A significant positive correlation between T specific antibody in serum and *H. pylori* IgG antibody level was found in *H. pylori*-infected normal blood donors ($P < 0.001$), but this correlation was not found in *H. pylori*-infected cancer patients. In summary, the cancer related T epitope is expressed in *H. pylori* and modulation of T antigen-specific immune response in *H. pylori*-infected individuals suggests that *H. pylori* infection may alter natural immune mechanisms against cancer.

INTRODUCTION

The Thomsen-Friedenreich (T) antigen (Galβ1,3GalNAcα/β-O-Ser/Thr) is a tumor-associated antigen expressed in a majority of human carcinomas. It has been found to be related to invasion and metastasis through interactions with surface lectins. T antigen has also been found to relate to prognosis.[1,2] Natural anti- T Ag antibodies are present in blood of every individual. This Ab level has been shown to be low in serum of patients with different malignancies[1,3] including gastric cancer[4] and in gastric premalignant conditions.[5] It is well established that chronic infection with *Helicobacter pylori* (*H. pylori*) is strongly associated with peptic ulcer disease, chronic gastritis and increased risk for gastric cancer.[6–8] It has been shown that both the *H. pylori* diversity and unique host response to *H. pylori* may contribute to a specific outcome of the infection.[9–15] However, there are still no reliable indicators to predict the sequelae of the infection at the individual level.

We have found recently that *H. pylori* infection is associated with an increased level of antibody to tumor-associated T antigen.[16] Moreover,the differences between various clinical groups (cancer vs. peptic ulcer disease) were demonstrated suggesting that the T specific immune response may be related to clinical outcome of the infection. In this study we present immunologic evidence that the cancer-related T-antigen is expressed in *H. pylori* and that the clinical isolates have different amounts of this antigen. In addition we found a positive correlation between the level of anti-T specific IgG antibody and the level of anti-*H. pylori* IgG in *H. pylori* infected blood donors.

MATERIAL AND METHODS

Subjects and Clinical Samples

Altogether 65 (42 men, 23 women) patients with histologically verified gastric carcinoma (median age-54.3 y) (47 *H. pylori-seropositive*) and 62 (29 men, 33 women) (38 *H. pylori-seropositive*) randomly selected blood transfusion donors (median age-47.8 y) were tested for *H. pylori* serology and T-specific IgG serum antibody. The *H. pylori* isolates from antral gastric mucosa biopsies of eight patients with benign gastric disorders and five *H. pylori* isolates from patients with gastric cancer (mucosa samples obtained at surgery) were further analysed for T antigen expression by immunoblotting and cell-ELISA.

All serum samples were stored at –20°C until analyzed.

Antibodies and Antisera

The following T-specific monoclonal antibodies (MAbs) were used (Table 1): human MAbs 5C7 (IgM) and TF1 (IgM) were a gift from B. Jansson (Lund, Sweden); murine MAb 9H8 (IgM) was from R. Sikut (Tartu, Estonia), murine MAb 3C9 (IgM) from Dr. H. Clausen (Copenhagen, Denmark), murine JAA-F11 MAb (IgG3) from K. Rittenhouse-Olson (Buffalo, USA). Anti-H type 2 murine MAb 19OLE (IgM) was a gift from Dr. J. Bara (Villejuif, France) and anti-CD43 (leukosialin carrying sialyl-Le^a MAb 3A1 (IgG1) from R. Sikut (Tartu, Estonia) were used as isotypic controls. All Mabs except JAA-F11 (purified) were hybridoma supernatants.

Other antibodies: Rabbit antiserum against heat shock protein (hsp60) of *H. pylori*[24] was kindly provided by I. Nilsson (Lund, Sweden) and rabbit antiserum to T hapten-bovine serum albumin conjugate[25] was a gift from Dr. K. Matta (Buffalo, USA).

Table 1. Binding Specificity of Monoclonal Antibodies as Reported in the Literature

Designation	Species & Isotype	Antigen Specificity	Authors (Reference)
TF1	Human IgM	T	Dahlenborg et al., 1997[17]
5C7	Human IgM	T	Jansson & Borrebaeck, 1992[18]
9H8	Murine IgM	T + peptide core	Sikut et al., 1996[19]
JAA-F11	Murine IgG3	T	Rittenhouse-Diakun et al., 1998[20]
3C9	Murine IgM	T	David et al., 1992[21]
19-OLE	Murine IgM	H type 2	Rouger et al., 1987[22]
3A1	Murine IgG1	CD-43 (leukosialin)	Sikut et al., 1999[23]

H. pylori Culture and Antigen Preparation

H. pylori strain NCTC 11637 was cultured on Gonococcal agar base/Campylobacter (GAB-CAMP) reference agar (Oxoid Ltd, Basingstoke, Hampshire, England) supplemented with 10% horse blood, selective supplements (Dent) and IsoVitaleX Enrichment (Oxoid) at 37°C for 3 days in plastic jars filled with a gas mixture of 5% O_2, 10% CO_2 and 85% N_2. The clinical *H. pylori* isolates from antral gastric mucosa were cultured in a similar way. A glycine cell surface membrane antigen extraction was performed according to Logan and Trust.[26]

H. pylori ELISA

Serum samples were examined by ELISA as described earlier.[27,28] In brief, 96-well flat bottom microtiter plates (Maxi Sorp, Nunc, Roskilde, Denmark) were coated with 0.1 mL of antigen (5 μg/mL in carbonate buffer pH 9.3) per well and incubated at 4°C overnight. Alkaline phosphatase conjugated goat anti-human IgG (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) and p-nitro-phenyl-phosphate (Sigma, St. Louis, MO) as substrate were used. A pool of human IgG (Kabi AB, Stockholm, Sweden) was placed in each ELISA plate as a positive control for 100 units and *H. pylori* seronegative reference serum was run for negative control. The relative antibody activity (RAA) values were calculated according to Blomberg et al.[29] RAA values ≤ 25 were regarded as negative. The subjects with 'gray' zone RAA values (26–40) were not included in this study.

Determination of Serum Anti-T IgG Antibodies by ELISA

These assays were carried out as described earlier.[4] Plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with synthetic T-hapten-polyacrylamide (PAA) conjugate (Synthesom, Munich, Germany; 10 mol% of carbohydrates) 5 μg/mL in carbonate buffer 50 mM Na_2CO_3/$NaHCO_3$ and 0.02% NaN_3, pH 9.6 or 1% BSA in PBS (control wells) at 4°C overnight. After washing three times with PBS-Tween (0.13 M NaCl, 8 mM NaH_2PO_4/KH_2PO_4, 3 mM KCl, 0.02% sodium azide, pH 7.2, 0.05% Tween 20) the plates were blocked with 0.15 mL of 1% BSA in PBS for 1 h at room temperature (RT) and washed in PBS-Tween. Serum (100 μL diluted 1:50 in PBS-Tween was added and incubated for 2 h at RT. The plates were then washed and bound IgG was detected with 100 μL of alkaline phosphatase conjugated goat anti-human IgG (Gibco, BRL, Life Technologies, Gaithersburg, MD, USA) diluted 1:1000 in PBS-Tween. Following an incubation of 90 min at RT and washing, the plates were developed with *p*-nitro-phenyl-phosphate (Sigma), 1 mg/mL in 1 M diethanolamine buffer,

pH 9.8 for 30 min and absorbance values at 405 nm were registered with Labsystem Multiscan MCC/340 (Finland). The optical density (O.D.) value of the control well (PBS-BSA) was subtracted from the values of the wells coated with the T conjugate. Each serum was analysed in duplicate. The intra-assay variations did not exceed 7%.

Sodium-Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

SDS-PAGE of glycine-extracted cell surface proteins of *H. pylori* NCTC11637 strain or clinical *H. pylori* isolates was performed in a 5% stacking and 12% polyacrylamide separating gel as described elsewhere.[28] After separation and blotting, the Immobilon P membrane (Millipore, Bedford, MA) was saturated as described by Rucheton et al., 1992[30] and incubated with patients sera (1:76) or rabbit antiserum (1:200) diluted in washing buffer or with MAb 1 μg/mL in PBS-0.5% Tween-20 (0.1% Tween-20 for MAbs of IgM isotype), overnight at +4°C under agitation. The strips were washed and incubated for 2 h at RT with alkaline phosphatase-labelled rabbit anti-human IgG (1:1000) (Gibco), or anti-human IgM (1:2000) (Dako A/S, Glostrup, Denmark), or rabbit anti-mouse or sheep anti-rabbit immunoglobulins (1:1000)(Dako) and developed with NBT/BCIP substrate (Sigma, St.Louis, Mo.). All extracts were tested in parallel under the same electrophoretic and blotting conditions, MAb dilutions etc. For semi-quantitative evaluation, the strips were scanned (Sharp image scanner JX-330) and the relative proportion of two major T antigen-related bands was calculated using ImageMaster TotalLab software (Amersham Pharmacia Biotech, Finland).

In blocking experiments, the strips were first treated with rabbit T-specific antiserum (1:100 in PBS-0.1% Tween-20) or with rabbit anti-hsp60 serum (1:200) or PNA (Sigma) 2 mg/mL in 0.05 M phosphate buffered saline pH 7.6-0.05% Tween-20 for 4 h at RT, washed, incubated with MAb TF1 or JAA-F11 overnight and developed with anti-human or anti-mouse Ig alkaline phosphatase conjugate and NBT-BCIP as described above.

H. pylori Cell-ELISA

Freshly prepared from 2 days culture *H. pylori* cells were fixed with 0.5% glutaraldehyde for 20 min at RT. After being blocked with 0.15 M glycine and 1% bovine serum albumin and washing 3x with PBS, 10^8 cells per tube in 0.1 mL of PBS were incubated with equal volumes of T antigen-specific MAb dilutions for 2 h at RT. Bacteria were then washed four times in PBS-0.05% Tween-20 and incubated with either alkaline phosphatase-conjugated rabbit anti-human IgM or, for murine MAb, with biotinylated

goat anti-mouse immunoglobulins (Dako, Denmark) for 1 h at RT followed by streptavidin-alkaline phosphatase conjugate (Dako) for 1 h. After 3 additional washes, the bacteria were incubated with p-nitro-phenylphosphate (Sigma, St.Louis, MO) for 30 min and the A^{405} of the supernatants was determined. Bacteria incubated with PBS instead of MAb were treated in the same way and served as the control. The assay was performed in duplicate.

Statistical Methods

The Student's t-test and Pearson two-tailed correlation were used for statistics. P value <0.05 were considered to be significant.

RESULTS

The human T antigen-specific MAbs TF1, 5C7, murine MAbs 3C9 and JAA-F11 revealed two major protein bands on blots of cell surface membrane extract of *H. pylori* NCTC 11637 strain: a dominant 58 kDa and a more weakly stained 68 kDa protein band (Fig. 1). One MAb (9H8) as well as nonrelated anti-H2 MAb 19OLE showed no binding. A different pattern of binding was observed for clinical isolates of *H. pylori*. All MAbs including 9H8 revealed both bands. However, the intensity of immunostaining as calculated on scans with ImageMaster TotalLab software (see Methods section) varies appreciably among isolates of different origin. The relative intensity for 68 kDa band was significantly higher ($p < 0.05$) in antigenic preparations from *H. pylori* isolates of patients with either gastric cancer ($46.7 \pm 5.1\%$, $n = 4$) or duodenal ulcers ($40.2 \pm 18.2\%$, $n = 3$) compared to strains derived from patients with chronic gastritis ($25.1\% \pm 18.3\%$, $n = 5$) or NCTC 11637 standard strain ($17.5 \pm 7.5\%$, six different preparations). These proportions were obtained with TF1 Mab but similar data were also observed for 5C7 MAb (not shown). Six preparations from different cultures of the NCTC 11637 *H. pylori* strain showed invariably low immunoreactivity for the 68 kDa glycoprotein. Two additional minor bands ($\sim$55 and 62 kDa) which bound TF1, 5C7 and 9H8 MAbs were revealed in one extract (chronic gastritis). Anti-H type 2 murine IgM MAb (19OLE) did not react with any bands in this area.

In blocking experiments, the PNA and T-specific rabbit antiserum almost completely abolished the binding of TF1 and JAA-F11 MAb (Fig. 2, lanes 3 and 7). Similar blocking effect of PNA and anti-T serum was observed for MAb 5C7 and JAA-F11. Anti-hsp60 serum revealed a dominant $\sim$58 kDa band (lane 4) and blocked the binding of T-specific TF1 MAb to 58 kDa band but not to 68 kDa band (lane 5). Both T-related bands did not bind the anti-H-type-2 mouse IgM MAb (lane 6).

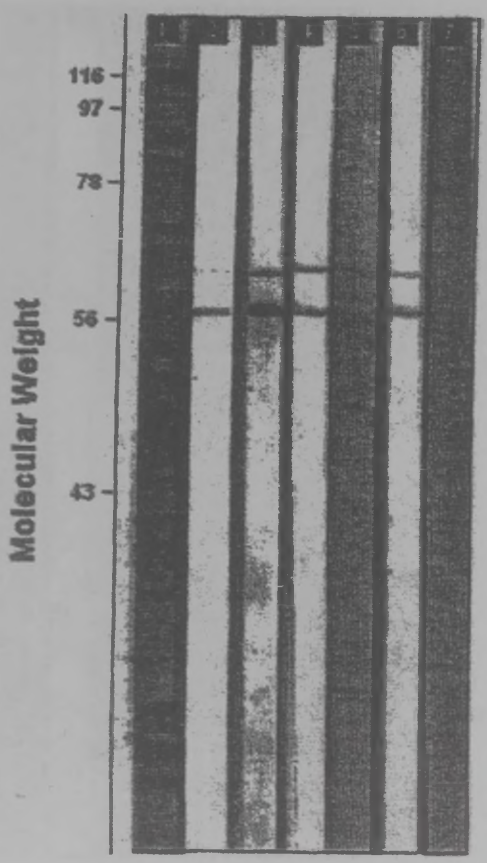

Figure 1. Western blot analysis of T antigen-specific monoclonal antibodies on cell surface membrane extract from *H. pylori* NCTC 11637 strain. Lane 1, *H. pylori* seropositive human serum (RAA – 94); Lane 2, human MAb 5C7; Lane 3, human MAb TF1; Lane 4, murine MAb 3C9; Lane 5, murine MAb 9H8; Lane 6, murine MAb JAA-F11; Lane 7, murine MAb 18OLE (anti-H type 2). Molecular mass markers (kDa) are at left.

In Cell-ELISA, binding of TF1 and 5C7 MAb to *H. pylori* cells was noted as was binding of JAA-F11 and 9H8 MAb (Fig. 3). All T-specific Mabs reacted in a dose-dependent manner. T-epitope unrelated anti-leukosialin MAb (3A1) showed very weak nonspecific binding irrespective of the dilution.

The T specific IgG antibody in serum did not differ significantly for *H. pylori*-seropositive and negative donors as measured by O.D. values (0.642 ± 0.096, $n = 38$ and 0.54 ± 0.053, $n = 24$, respectively; $t = 0.94$, $p > 0.5$). When compared to blood donors, significantly lower Ab was found in patients with gastric cancer for both the *H. pylori*-seropositive as measured by O.D. values (0.359 ± 0.030, $n = 42$, $t = 3.0$, $p < 0.002$) and *H. pylori*-negative (0.227 ± 0.038; $n = 18$, $t = 4.6$, $p < 0.001$) subgroup. The *H. pylori*-seropositive patients with gastric cancer showed a significantly higher anti-T Ag response than seronegative cancer patients ($t = 2.72$, $p < 0.02$).

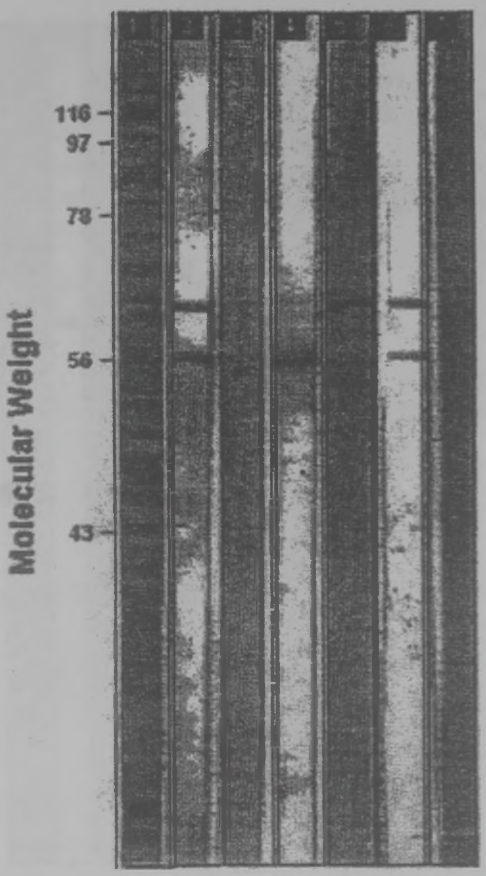

Figure 2. Immunoblot analysis using clinical isolate of *H. pylori* (chronic gastritis) as antigen: immunostaining with T antigen-specific Mabs, and blocking experiments. Lane 1, *H. pylori* seropositive human serum (RAA 112); Lane 2, human MAb TF1; Lane 3, human MAb TF1, blocked with PNA; Lane 4, rabbit antiserum to hsp of *H. pylori*; Lane 5, human MAb TF1, blocked with rabbit antiserum to HSP of *H. pylori*; Lane 6, murine MAb JAA-F11; Lane 7, murine MAb JAA-F11, blocked with rabbit antiserum to T antigen. Molecular mass markers (kDa) are at left.

In blood donors, a level of IgG T-specific antibodies (O.D. values) positively correlated with anti-*H. pylori* IgG antibody level (RAA values) (Table 2). In contrast, no correlation between T-specific antibody and *H. pylori* RAA values was observed in the patients with gastric cancer irrespective of *H. pylori* status.

DISCUSSION

The data show that several T antigen-specific human and murine monoclonal antibodies of the IgM and IgG isotype reacted with two

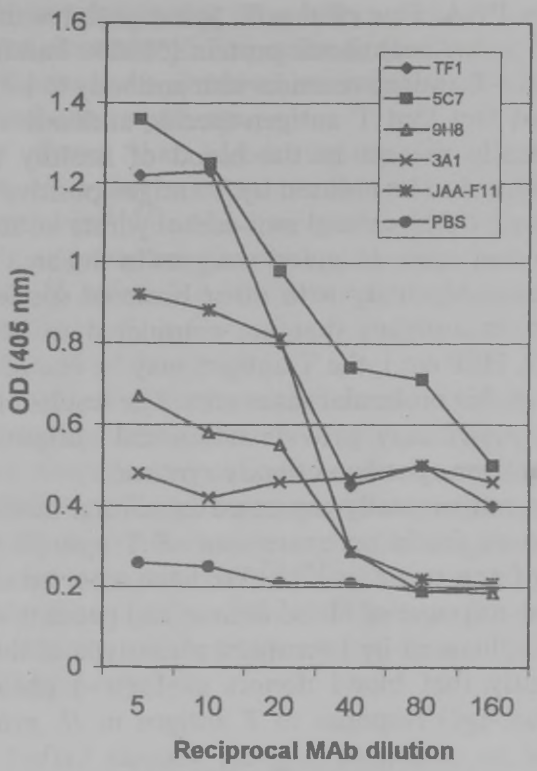

Figure 3. Binding of T antigen-specific monoclonal antibodies to *H. pylori* NCTC 11637 strain cells as assessed by cell-ELISA with 10^8 bacteria. OD (405 nm), optical density at 405 nm.

dominant proteins that are present in cell surface membrane extract of *H. pylori*. Both immunoblotting and cell-ELISA showed comparable results. The specificity was shown by the fact that the binding of T antigen-specific antibodies was blocked with polyclonal antiserum to T disaccharide-BSA

Table 2. Correlation Between a Level of Serum IgG Antibodies to T Antigen and Anti-*H. pylori* Antibodies in *H. pylori*-Infected and Uninfected Blood Donors and Patients with Gastric Cancer

	H. pylori Seropositive			*H. pylori* Seronegative		
Groups and Compared Parameters	n	r	P	n	r	P
Donors anti-T IgG vs. anti-*H. pylori* IgG (RAA)	38	0.57	<0.001	24	-0.046	0.80
Cancer Patients: anti-T IgG vs. anti *H. pylori* IgG (RAA)	47	-0.13	0.36	18	0.089	0.74

conjugate and by PNA. One of the *H. pylori* proteins that contains the T epitope is the *H. pylori* heat shock protein (58 kDa band) as shown by the blocking of the anti-T antigen reaction with antibody to hsp60 of *H. pylori*. It is well established fact that T antigen-specific antibodies of IgG and IgM isotype are normally present in the blood of healthy individuals. Their synthesis is considered to be induced by T antigen positive resident intestinal flora[31,32] like many other natural anti-carbohydrate antibodies.[33]

It is known that some *H. pylori* antigens in the area of 43–70 kDa are responsible for cross-reactivity with other bacterial species.[34,35] It appears that, in addition to antigens that are considered to be shared by other bacteria (flagellar, HSP etc.), the T antigen may be one of the cross-reacting epitopes located in this molecular mass area. The results of the present study suggest that *H. pylori* may provide additional antigenic stimuli for the activation of T antigen-specific antibody synthesis.

T epitope is not normally expressed in normal human cells. The only exception known so far is an expression of T epitope in surface gastric epithelium cells of non-secretors.[36,37] We have reported earlier that natural T specific immune response of blood donors and patients with gastric cancer was appreciably influenced by Lewis(a,b) phenotype of the host.[4] We have also found recently that blood donors of Le(b−) phenotype revealed a significantly higher IgG response to T antigen in *H. pylori*-infected blood donors compared to uninfected subjects whereas Le(b+) secretors did not show such difference.[16]

The differences in the binding of 9H8 MAb between NCTC 11637 *H. pylori* strain and clinical isolates of *H. pylori*, as well as the variations in immunostaining of 58 and 68 kDa proteins with other MAbs, suggest that T epitope expression varies appreciably between the strains. This might be explained by polymorphism in glycosylation patterns of gastric glycoconjugates that are closely related to ABH and Lewis phenotype of the host. Interestingly, a standard NCTC 11637 *H. pylori* strain differed in that practically no expression was observed for 68 kDa protein band of *H. pylori*. It has been shown that glycosylation of gastric mucins may be altered by *H. pylori*.[38,39] Barresi et al.,[40] found a higher expression of T antigen in *H. pylori*-positive mucosa samples: 16% vs. 5% in uninfected patients. The glycosidase activity of *H. pylori*[41] may be involved. Possible association between the T antigen-related proteins of *H. pylori* and particular gastric disease requires further validation. There is more pronounced expression of 68 kDa protein in *H. pylori* strains derived from patients with gastric cancer and peptic ulcer disease than in individuals with the more mild gastritis, thus T Ag expression on this molecule may be related to more severe pathology.

A difference in a level of T specific antibodies between the infected and uninfected subjects was demonstrated for the patients with gastric cancer. However, a positive correlation between anti-T IgG levels (O.D.) and anti-*H. pylori* specific IgG antibody was found only in *H. pylori*-seropositive blood

donors group. No correlation was observed in patients with cancer possibly due to a significant decrease of T specific antibody levels in a majority of patients.[4] No difference was observed in anti-*H. pylori* IgG (RAA) values between donors and cancer patients.

It has been shown that higher density of antral gastric mucosa colonization with *H. pylori* was associated with a higher IgG immune response to the organism[42,43] and a stronger local inflammatory response to *H. pylori*.[13,14] We suggest that the correlation we found between the IgG immune response to *H. pylori* (RAA) and the level of T specific IgG antibody may reflect a density of *H. pylori* colonization or a degree of T epitope expression in a given *H. pylori* strain.

Our findings are the first evidence that cancer-related autoimmunogenic T epitope is expressed in surface membrane glycoconjugates of *H. pylori* and associated with an alteration of natural immune response to T antigen in infected subjects. Different *H. pylori* strains seem to express the T epitope to a different degree with some relation to a particular gastric pathology. The data suggest that, depending on the ability of the host to elicit an acquired immune response to the *H. pylori*-derived T epitope, the *H. pylori* infection may alter natural immune mechanisms against cancer thus modulating the risk associated with this infection.

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VIII

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Better Survival of *Helicobacter pylori* Infected Patients with Early Gastric Cancer Is Related to a Higher Level of Thomsen-Friedenreich Antigen-Specific Antibodies

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ABSTRACT

The survival of patients with histologically verified gastric carcinoma at stage I (n = 44) and stage II (n = 43) was analysed by the Kaplan-Meier method depending on *H. pylori* serological status and a level of IgG and IgM antibody to tumor-associated Thomson-Friedenreich antigen (T Ag). In cancer patients at stage I, significantly better survival for *H. pylori* seropositive patients was observed compared to *H. pylori* seronegative patients (median ± SE survival time: 60.0 ± 3.8 mths and 37.0 ± 7.8 mths, respectively; $P < 0.0004$, log-rank test). Patients with higher level of T Ag-specific IgG antibody (strong responders) showed significantly and dramatically better ($P < 0.00001$) survival rate than weak responders. However, an association of better survival with a higher level of anti-T antibody level was limited to the *H. pylori* seropositive patients exclusively ($P < 0.00001$) with no difference for *H. pylori* seronegative group of patients. The level of IgM anti-T Ag antibody was not significantly related to the survival of patients at both stages of the disease, though better survival was noted in *H. pylori* seropositive IgM strong responders at ~40–60 months of observation. Statistically insignificant associations between survival and *H. pylori* status or anti-T antibody levels were also observed in a group of gastric

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cancer patients at stage II. In summary, the survival of patients with early gastric cancer (stage I) is significantly better in *H. pylori* seropositive patients, and this phenomenon may be in part explained by up-regulation of T Ag-specific IgG immune response in *H. pylori* infected individuals.

Key Words: *Helicobacter pylori*; Thomsen-Friedenreich antigen; T antigen-specific immune response; Gastric cancer; Survival.

INTRODUCTION

Helicobacter pylori (H. pylori) infection is closely related to the pathogenesis of various gastroduodenal diseases including gastric cancer. Several factors have been considered to influence the specific clinical outcome of the infection including *H. pylori* diversity, unique host response, evironmental exposures or the combination of several factors in genetically susceptible host (Blaser and Berg, 2001; Ernst et al., 2001; Solnic and Schauer, 2001). *H. pylori* elicits a strong systemic and mucosal immune response both humoral and cell-mediated. The proinflammatory cytokine cascade induced by *H. pylori* is an important factor leading to mucosal damage, atrophy and genetic instability of the gastric epitthelial cells (Bamford et al., 1998; Nardone et al., 1999; Ren et al., 2000). However, few strong associations have been demonstrated regarding the disease type-specific changes in immune response to *H. pylori* (Bontkes et al., 1992, Pineros et al., 2001). One of the reasons is that immune response to *H. pylori* is highly polymorphic in terms of its strength, profile and degree of inflammation which are all dependent on host polymorphism.

Evidence is accumulating that an ongoing immune stimulation by *H. pylori* antigens may lead to the autoimmune reactions of various specificities (Faller et al., 2000; Negrini et al., 1996; Vorobjova et al., 2000). Blood group related antigens such as Lewis type 1 and type 2, have been shown to be targets (Appelmelk et al., 1996; Kurtenkov et al., 1999b). We have reported previously that, in different groups of patients with gastroduodenal pathology including gastric cancer, *H. pylori* infection was associated with an enhanced humoral immune response to tumor-associated blood group related Thomsen-Friedenreich antigen (T Ag) (Galβ1,3GalNAcα/β-O-Ser/Thr (Klaamas et al., 2002a). This impact was influenced by Lewis phenotype of the host and in part disease-type specific. Recently we have presented an immunological evidence that *H. pylori* itself expresses T Ag with an appreciable variation in expression of T Ag-related glycoproteins in *H. pylori* isolates derived from patients with different gastroduodenal diseases (Klaamas et al., 2002b).

The expression of T antigen in cancer cells is related to biological behaviour of tumor and the prognosis of cancer patients (Baldus and Hanish, 2000; Springer, 1984; Takanami, 1999). T antigen may be related to metastasis through interaction of this saccharide on the tumor cell with receptors on metastatic sites (Glinsky et al., 2001). An increase of antibodies against T and Tn epitopes after active immunotherapy of cancer with mucin-type vaccines containing these epitopes was associated with a more favourable prognosis of patients with cancer (Livingston and Ragupathi, 1997; Livingston et al., 1997; Maclean et al., 1992). In addition, low level of T-specific antibodies is associated with an increased risk for cancer (Springer, 1984).

To evaluate the possible clinical relevance of *H. pylori* infection-induced modulation of humoral immune response to T antigen, we analyzed the survival time of patients with early gastric cancer depending on their *H. pylori* serologic status and the level of T Ag-specific antibodies in their blood before surgery. We found that the *H. pylori* seropositive patients at stage I had significantly better survival compared to *H. pylori* seronegative patients and this effect was related to a higher level of T Ag-specific IgG antibodies in *H. pylori* infected patients.

MATERIAL AND METHODS

Patients with histologically verified gastric carcinoma at stage I (n = 44, median age 62yrs, male/female ratio-1.58) and stage II (n = 43, median age 64yrs, male/female ratio-1.39) were included in the study. Tumor stage was evaluated according to the international classification of malignant tumors (Sobin and Wittekind, 1997). The patients were tested for H. pylori serologic status and the level of anti-T Ag-specific IgG and IgM antibody. Venous blood was taken before surgery and serum was stored at −20 °C untill tested.

H. pylori Serology

H. pylori serologic status was determined by ELISA as described elsewhere (Klaamas et al., 1997). The relative antibody activity (RAA) value less than or equal to 25 was considered as seronegativity.

Determination of T Ag Specific Antibodies

The level of T Ag specific IgG and IgM antibodies in serum was tested by ELISA using synthetic T disaccharide-polyacrylamide conjugate (Synthesom, Germany) as antigen (Kurtenkov et al., 1999a). Serum dilution was 1:50 and 1:500 for IgG and IgM antibody determination, respectively. Depending on the optical density (O.D.) values for IgG and IgM antibodies, the patients were divided into 'strong' and 'weak' responders. The O.D. value more than 0.26 and 0.3 for IgM and IgG antibody level respectively, were considered as strong response. Patients with O.D. values less than or equal to these cut-off limits were considered as weak responders. These cut-off limits were calculated on the basis of the O.D. values distribution for the whole group of cancer patients tested, and allowed the best discrimination between the patients with low and high O.D. values.

Statistics

The cumulative survival of cancer patients was estimated by the Kaplan-Meier method, and the resulting curves were compared by use of the log-rank test (SPSS software, version 10.0.5). Mean and median survival time with the standard error and 95% confidence interval was calculated for every group of patients divided by *H. pylori* status and/or anti-T Ag specific antibody level. Statistical tests were two-sided and the

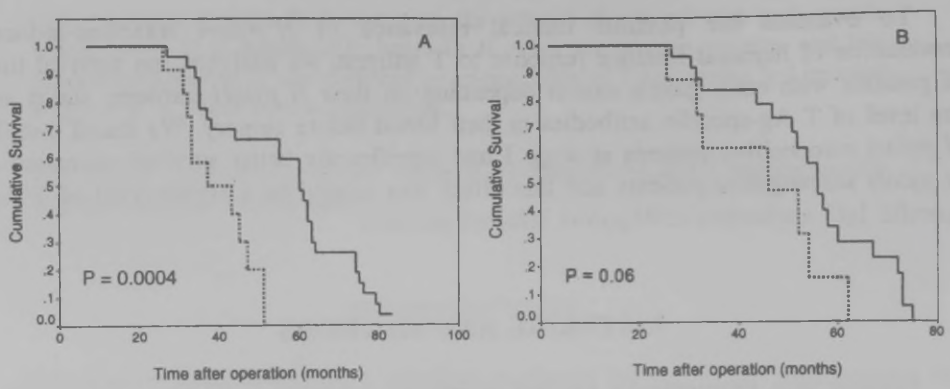

Figure 1. Cumulative survival plots (Kaplan-Meier) of *H. pylori* seropositive and *H. pylori* seronegative gastric cancer patients. The survival rate of gastric cancer patients in relation to *H. pylori* serologic status. A – stage I (n = 44), B – stage II (n = 43). Dark line - *H. pylori* seropositive patients; Dotted line - *H. pylori* seronegative patients.

difference in survival between the groups was considered significant if $P < 0.05$. The proportions of strong responders were compared by the Fisher's exact test.

RESULTS

The cumulative survival of patients in relation to *H. pylori* serologic status and/or a level of T Ag-specific IgG and IgM antibodies (O.D. values) is presented in Figures 1–4. A significantly better survival was observed in *H. pylori* seropositive patients at stage I of the disease compared to *H. pylori* seronegative ones (Figure 1A). The survival time (median ± SE) was 60.0 ± 3.8 and 37.0 ± 7.8 months for *H. pylori* positive and *H. pylori* negative patients, respectively (P = 0.0004). A similar trend was noted for the patients at stage II (Figure 1B): 56 ± 2.7 and 46 ± 11.3, P = 0.06).

Table 1. The proportions of gastric cancer patients with higher level of IgG and IgM antibody to T antigen (strong responders) in *H. pylori* seropositive and *H. pylori* seronegative patients.

	Anti-T IgM antibody		Anti-T IgG antibody	
Stage	*H. pylori* (+)	*H. pylori* (–)	*H. pylori* (+)	*H. pylori* (–)
Stage I	13/30[a] (43.3%)	7/14 (50.0%)	18/26 (**69.2%**)[b]	3/11 (27.3%)
Stage II	15/28 (53.6%)	4/13 (30.8%)	12/22 (54.5%)	3/11 (27.3%)

H. pylori (+) and *H. pylori* (–) : *H. pylori* seropositive and *H. pylori* seronegative patients, respectively.

[a]The number of patients with high level of antibody/the number of tested subjects. In brackets – the percentage of strong responders.

[b]Significantly higher proportion of strong IgG responders compared to *H. pylori* seronegative patients of stage I (P = 0.023, Fisher's exact test).

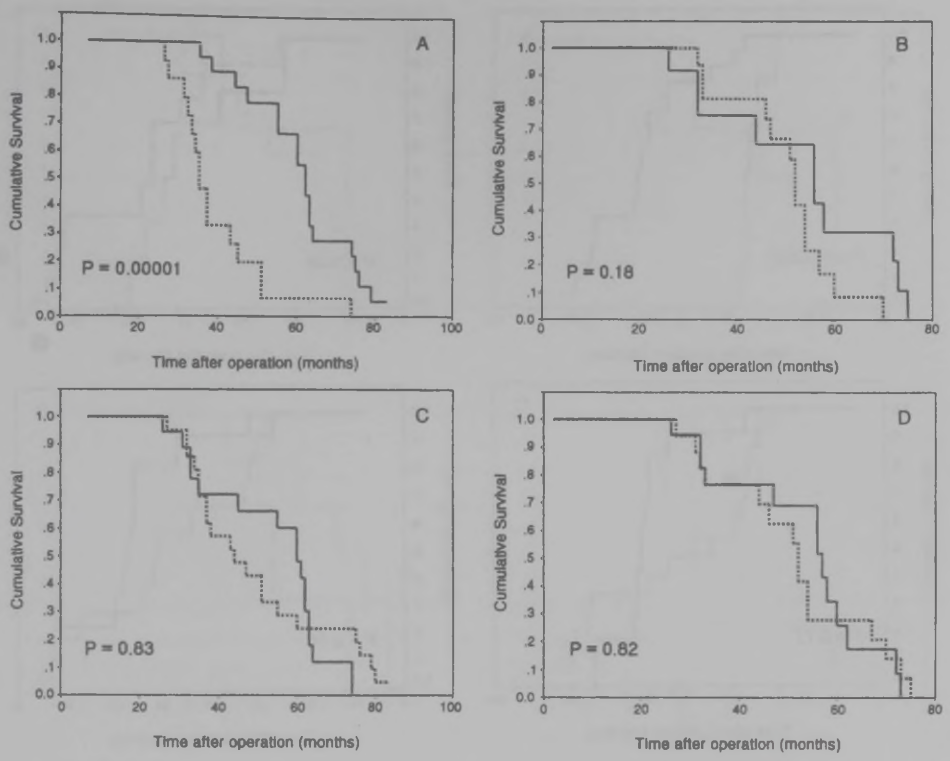

Figure 2. Cumulative survival plots (Kaplan-Meier) of gastric cancer patients in relation to anti-T Ag IgG (A,B) and IgM (C,D) antibody level calculated irrespective of *H. pylori* status. A – stage I, IgG antibody (n = 37); B – stage II, IgG antibody (n = 34); C – stage I, IgM antibody (n = 44); D – stage II – IgM antibody (n = 43). Dark line – strong responders (O.D.values above cut-off limit); Dotted line – weak responders (O.D. values below or equal to the cut off limit).

A significant over-representation of strong IgG responders was observed among *H. pylori* seropositive stage I patients compared to *H. pylori* seronegative ones (Table 1). Similar trend was found in patients at stage II, but the differences did not reach the statistical significance (P = 0.13). The same was true for IgM T antibody level (P = 0.12).

A comparison of patient's survival in relation to anti-T Ag antibody levels, calculated irrespective of *H. pylori* status, showed that stage I patients with higher level of T Ag specific IgG antibody had significantly better survival compared to those with low level of antibody (weak responders): median survival time 62.0 ± 2.1 and 35 ± 1.4 months, respectively; P = 0.00001 (Figure 2A). However, only *H. pylori* seropositive strong IgG responders were better survivors (P = 0.00001), (Figure 3, A) whereas strong IgG responders in *H. pylori* seronegative group were not (P = 0.96)(Figure 4A). Similar trends were observed in a group of patients at stage II (Figures 3B, 4B).

A slight tendency for better survival survival was also shown for *H. pylori* seropositive IgM strong responders compared to IgM weak responders in this group (Figure 3C,D). The difference concerned a period of ~40–60 months of observation and disappeared later. Interestingly, in contrast to *H. pylori* seropositive patients, a

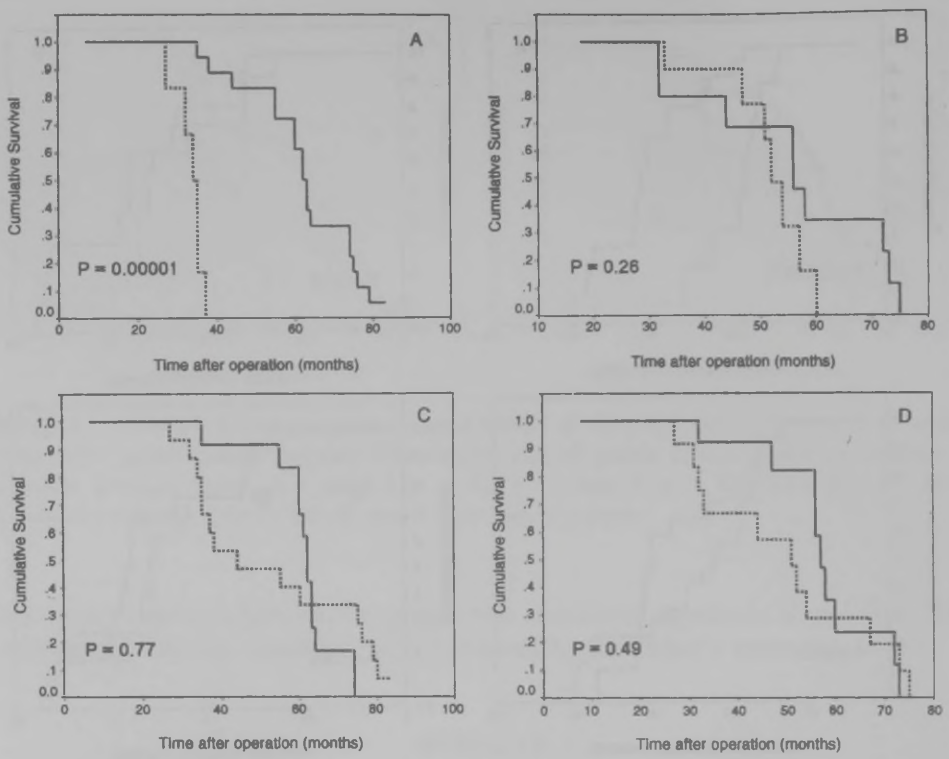

Figure 3. Cumulative survival plots (Kaplan-Meier) of *H. pylori* seropositive gastric cancer patients depending on a level of T Ag-specific IgG (A,B) and IgM (C,D) antibodies. A – stage I, IgG antibody (n = 26); B – stage II, IgG antibody (n = 22); C – stage I, IgM antibody (n = 30); D – stage II, IgM antibody (n = 30). Dark line – strong responders; Dotted line – weak responders.

slightly better survival was found for *H. pylori* seronegative IgM weak responders at both stages of the disease (P = 0.12–0.18), (Figure 4C,D). Nevertheless, the *H. pylori* seropositive strong IgM responders (Figure 3C,D) showed significantly better median survival time compared to *H. pylori* seronegative IgM weak responders (Figure 4C,D): 60.0 ± 4.02 vs. 33.0 ± 1.15 mths, P < 0.001, and 57.0 ± 1.4 vs. 32.0 ± 3.50 mths, P < 0.001) for stage I and stage II, respectively.

DISCUSSION

The data suggest that *H. pylori* positive serologic status is associated with a better long-term survival of patients with early gastric cancer. Comparing the survival irrespective of *H. pylori* status (Figure 2), a high level of T Ag specific IgG antibodies was also related to a significantly better survival of cancer patients at stage I. However, the difference in survival between the patients with high and low level of T Ag IgG antibody was found only for *H. pylori* seropositive individuals (Figure 3). Some

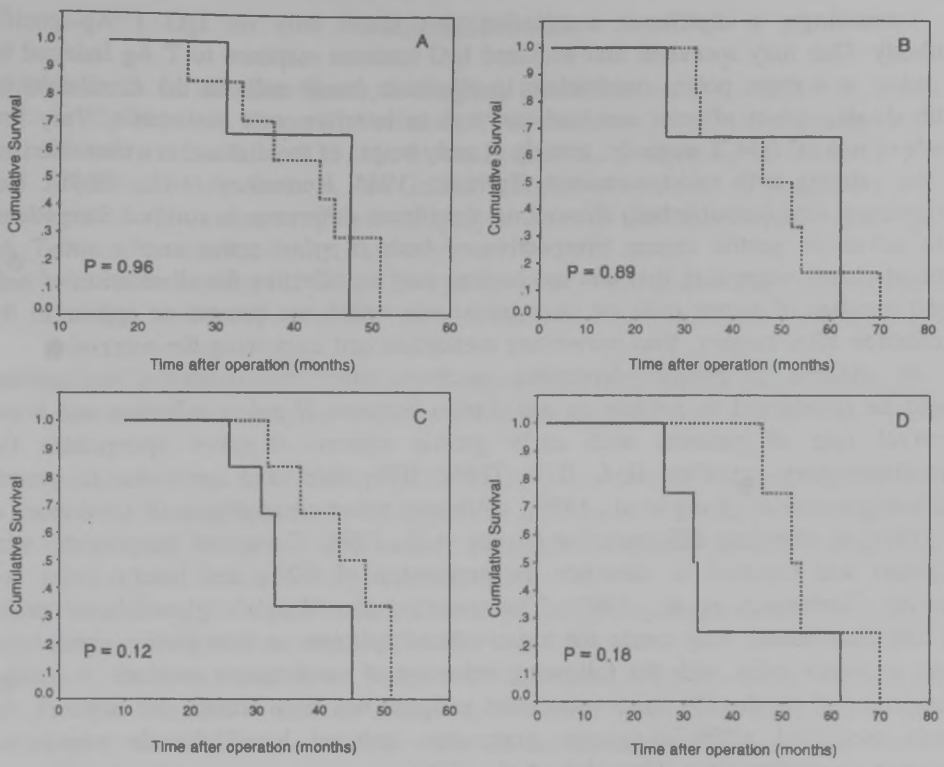

Figure 4. Cumulative survival plots (Kaplan-Meier) of *H. pylori* seronegative gastric cancer patients depending on a level of T Ag-specific IgG (A,B) and IgM (C,D) antibodies. A – stage I, IgG antibody (n = 11); B – stage II, IgG antibody (n = 13); C – stage I, IgM antibody (n = 14); D – stage II, IgM antibody (n = 13). Dark line – strong responders; Dotted line – weak responders.

additive effect on the survival observed in *H. pylori* seropositive IgG strong responders compared to *H. pylori* seropositive status alone (Figure 1A) suggest that both high level of T-antibody and *H. pylori* seropositive status are involved. The absence of beneficial effect on the survival in *H. pylori* seronegative strong responders indicates that the presence of *H. pylori* infection seems to be an important independent prognostic factor. Since *H. pylori* seropositive patients had a higher proportion of strong responders, it appears that higher level of T Ag-specific IgG antibody associated with *H. pylori* infection may be in part responsible for a better survival observed in H. pylori seropositive patients. In contrast, the *H. pylori* seronegative patients did not reveal any appreciable differences in the survival.

Thus, in general for *H. pylori* seropositive patients individuals with higher level of T Ag antibody had better survival. In *H. pylori* seronegative group, a level of IgG T antibody was not significantly associated with the survival. Moreover, median survival time values in *H. pylori* seronegative group (37 ± 7.9mths, calculated irrespective of T antibody level, Figure 1) were similar to those of weak IgG responders in *H. pylori* seropositive group (34.0 ± 1,2mths; Figure 3A). This suggests that both *H. pylori* status and high level of T antibody was beneficial for the patient's survival.

Interestingy, a significant association was found only for IgG T Ag-specific antibody. One may speculate that acquired IgG immune response to T Ag induced by *H. pylori* is a more potent mechanism to eliminate tumor cells in the circulation by antibody-dependent effector mechanisms, and to interfere with metastasis. Very low levels of natural IgM T antibody, already at early stages of the disease, is a characteristic of the patients with various cancers (Springer, 1984, Kurtenkov et al., 1999a). Our preliminary data (unpublished) showed no significant difference in survival for patients with advanced gastric cancer irrespective of both *H. pylori* status and/or anti-T Ag antibody level suggesting that this mechanism may be effective for elimination of only small number of tumor cells or micrometastasis which are present or appear in the circulation after surgery, thus preventing metastasis and improving the survival.

In addition to antibody-dependent reactions, other immunological mechanisms might be considered to explain an association between *H. pylori* infection and better survival rate of patients with early gastric cancer. *H. pylori* upregulates the proinflammatory cytokines IL-6, IL-8, TNFα, IFNγ that have anti-tumor or growth inhibiting potential (Kang et al., 1999), inhibitory effect on angiogenesis (Jenkinson et al., 2002) or stimulate differentiation (Arany et al., 1999). Contact of lymphocytes with *H. pylori* was reported to stimulate the production of IFN-γ and natural killer cell activity (Tarkkanen et al., 1993). Theoretically, the *H. pylori* glycosidases and/or glycosyltransferases may create the tumor-related epitopes on host gastric glycoconjugates or tumor cells, with the following induction of autoimmune response. A similar production of saccharide tumor-associated antigens has been shown, for instance, for tumor-associated alphaGal-epitope expression induced by *Klebsiella pneumonia* enzymes on erythrocytes (Hamadeh et al., 1996).

A growing body of evidence suggests that *H. pylori* infection may actually have some beneficial effects. The carriage of the more virulent CagA-positive strain was shown to be associated with a reduced risk of esophageal and gastric cardial adenocarcinoma and of the gastroesophageal reflux disease (Chow et al., 1998; Kuipers, 1999; Loffeld et al., 2000). *H. pylori* infection may protect against diarrheagenic gastrointestinal infections in children (Rothenbacher et al., 2000). Our data shows better survival of *H. pylori* infected patients with gastric cancer and indicates that, in some situations, this infection may be beneficial for the host. Therefore, eradication of H. pylori becomes more controversial, especially in individuals of older age.

Our findings are important and are the first evidence that *H. pylori* seropositive patients with early gastric cancer have better survival than *H. pylori* seronegative patients, and that this impact is related to the up-regulation of T Ag specific immune response by *H. pylori* infection. This further supports the idea that stimulation of immune response to T Ag may be beneficial for the host in a situation when primary tumor is removed. Since infection with *H. pylori* is relatively common, it may be considered as a good model to study the impact of infections on anti-tumor immunological mechanisms.

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1983–1990	The Second Moscow State Medical Institute, medico-biological faculty
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Special courses

International Summer School, Palanga, Lithuania, 1993.
Immunochemical methods, Lund University, Lund, Sweden, 1994.
Course in immunotechnology, Biotechnology Research Foundation, Lund, Sweden, 1995.
H.pylori infection: clinical significance, diagnosis and treatment. Tartu University, Tartu, 1996
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Knowledge of languages: Estonian, Russian, English.

Professional employment

1990–1992 Estonian Institute of Experimental and Clinical Medicine (IECM) Department of Experimental Oncology, senior technician
1992–1995 IECM, Department of Experimental Oncology, junior research scientist
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Scientific work

Major areas of research: (1) *H.pylori* and gastric cancer: the immune response to *H.pylori* infection in gastric cancer patients; *H.pylori* infection and the mechanisms of anti-tumor natural immune response. (2) Tumor immunology: tumor associated antigens.

Member of the Estonian Society of Immunology (since 1992) and the Baltic Immunological Society (since 1993).

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Immunotehnoloogia kursus, Biotechnology Research Foundation, Lund, Rootsi, 1995.
H.pylori infektsiooni kliiniline tähendus, diagnoosimine ja ravi. TÜ arstide ja proviisorite täienduskeskus, Tartu 1996
Moodsa epidemioloogia kursus, TÜ tervishoiu instituut, 2003

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1995–2003	EKMI, onkoloogia osakond, teadur
alates 2003	Tervise Arengu Instituut, onkoloogia osakond, teadur

Teadustegevus

Põhilised uurimissuunad: (1) *H.pylori* ja maovähk: anti-*H.pylori* immuunvastus maovähihaigetel; *H.pylori* infektsioon ja loomuliku kasvajavastase resistentsuse mehhanismid. (2) Kasvaja immunoloogia: kasvajaseoselised antigeenid.

Eesti Immunoloogide (alates 1992) ja Balti Immunoloogide Seltsi (alates 1993) liige.

Saadud kaks granti: (1) *Helicobacter pylori* infektsioon maovähihaigetel: mikroobist ja peremehest sõltuvate tegurite immunoloogiline uuring. ETF grant nr. 2697, 1997–1999; (2) *Helicobacter pylori* infektsiooni süsteemne mõju kasvajaseoselistele süsivesikantigeenidele suunatud loomulikule immuunvastusele maovähi ja mao krooniliste haigustega patsientidel. ETF grant nr. 4217, 2000–2002

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