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Gastrointestinal Mucosal Protective Mechanisms

Mudolatory Effects of Heliobacter pyroli *on the Gastric Mucus Gel Barrier and Mucosal Blood Flow* in vivo

BY

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ABSTRACT
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The gastrointestinal mucus gel layer and blood flow are two important mechanisms for protection at the pre–epithelial and sub–epithelial levels, respectively. *Helicobacter pylori* might circumvent these mechanisms and elicit a chronic inflammatory response with consequent ulcers in the stomach and duodenum. In this thesis, the physical state and properties of the adherent mucus gel layer was studied from the stomach to colon. Furthermore, the acute and chronic effects of *H. pylori* on the integrity of the mucus gel layer and mucosal blood flow were studied in the anesthetized rat.

A translucent mucus gel covers all studied segments of the gastrointestinal tract during fasting conditions, with the thickest layers in the colon and ileum. Carefully applied suction revealed that the mucus gel was a multi-layered structure comprising a firmly adherent layer covering the mucosa, impossible to remove, and a loosely adherent upper layer. The firmly adherent layer was thick and continuous in the corpus (80µm), antrum (154µm) and colon (116µm), but thin (<20µm) and discontinuous in the small intestine.

Following mucus removal, a rapid renewal of the loosely adherent layer ensued. The highest rate was observed in the colon with intermediate values in the small intestine. Mucus renewal in the stomach was attenuated on acute luminal application of water extracts from *H. pylori* (HPE). In animals with a chronic *H. pylori* infection the mucus renewal rate was unaffected, but the total gastric mucus gel thickness was reduced and the mucus secretory response to luminal acid (pH1) attenuated in the antrum.

HPE from type I strains acutely reduced corporal mucosal blood flow, measured with laser–Doppler flowmetry, by approximately 15%. The reduction in blood flow was mediated by a heat stable factor other than VacA and CagA. Inhibition of endogenous nitric oxide production with N^{ω}–nitro–L–arginine augmented the decrease. However, ketotifen, a mast cell stabilizer, completely attenuated the effect of the extract as did the platelet activating factor (PAF) receptor-antagonist, WEB2086, thus depicting a detrimental role for the microvascular actions of PAF.

Key words: Mucus gel layer, mucosal blood flow, mucus thickness, chronic infection, rat, platelet activating factor, mast cell, nitric oxide, nitric oxide synthase, ketotifen, intra–vital microscopy, microelectrode, laser–Doppler flowmetry, rat.

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To my family

”Those who are althogether unaccustomed to research
are at the first exercise of their intelligence befogged and blinded
and quickly desist owing to fatigue and failure of intellectual power,
like those who without training accept a race.
But one who is accustomed to investigation, worming his way and turning
in all directions, does not give up the search, I will not say day or night,
but his whole life long. He will not rest but will turn his attention
to one thing after another which he considers relevant to the subject
under investigation until he arrives at the solution of his problem”.

Erasistratus of Julis (330–250 B.C.)

CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Atuma C, Strugala V, Allen A, Holm L. The adherent gastrointestinal mucus gel layer: Thickness and physical state *in vivo*. Submitted Am J Physiol 2000.

II Atuma C, Johansson M, Li H, Engstrand L, Holm L. Acute and chronic effects of *Helicobacter pylori* on the gastric mucus gel *in vivo*. Manuscript.

III Atuma C, Engstrand L, Holm L. Extracts of *Helicobacter pylori* reduce gastric mucosal blood flow through a VacA- and CagA-independent pathway in rats. Scand J Gastroenterol 1998;33:1256–61.

IV Atuma C, Engstrand L, Holm L. *Helicobacter pylori* extracts reduce gastric mucosal blood flow by a nitric oxide-independent but mast cell- and platelet-activating factor-dependent pathway in rats. Scand J Gastroenterol 1999;34:1183–9.

ABBREVIATIONS

ADMA	Asymmetric dimethyl arginine
bw	Body weight
CagA	Cytotoxin associated gene A
Cag PAI	Cag Pathogenicity island
CFU	Colony-forming units
CGRP	Calcitonin gene related peptide
EPE	*E. Coli* water extract
HCl	Hydrochloric acid
HPE	*H. pylori* water extract
IU	International units
L–NNA	N^{ω}–nitro–L–arginine
LDF	laser–Doppler flowmetry
LPS	Lipopolysaccharide
MAP	Mean arterial blood pressure
NO	Nitric oxide
NOS	Nitric oxide synthase
PAF	Platelet activating factor
PFU	Perfusion units
PGE_2	Prostaglandin E_2
SP	Substance P
VacA	Vacuolating cytotoxin A

INTRODUCTION

Gastrointestinal function and protective mechanisms have intrigued physiologists for centuries (reviewed in Modlin, 1995, Allen and Garner, 1980). In particular the question of how the stomach and intestine avoid being damaged by endogenous aggressors such as digestive enzymes, acid and bile has been studied. The gastrointestinal tract is open toward the outer milieu and as such has to afford protection against noxious substances and microorganisms ingested together with the food. It is the bodies largest immunological organ infiltrated with inflammatory cells and areas of lymphoid tissue (e.g. peyer's patches in the intestine). Naturally, there is a balance between aggressive forces and protective mechanisms, thereby maintaining the integrity of the mucosa. In case of an increased aggressor challenge, the defense mechanisms need to be upregulated to avoid pending injury or invasion of the mucosa. In peptic ulcer disease these protective mechanisms have been breached directly by toxic substances in the lumen, e.g. non–steroid anti–inflammatory drugs (NSAIDs), or by the interaction of the microorganism *Helicobacter pylori* and its cytotoxic products, with the mucosa. The recorded history of these disturbances dates back to the days of Hippocrates (≈350 B.C.) who first described the symptoms of gastric disease, "*Diocles of Carystos*".

Hunter's "*living principle*" was the first hypothesis on how the stomach was protected from autodigestion. In 1772, he ascribed the protection to an adequate continuous blood flow through the tissue. Virchow made a contribution to this hypothesis in 1853, which involved the acid-neutralizing power of the blood flow. Thus, in ulcer patients the supply of this alkaline blood was thought to be restricted enabling back-diffusing acid to damage the mucosa. Indeed, in the 1820's it was convincingly shown that the stomach actually produced hydrochloric acid after the work by Prout (1823) and Beaumont (1826–33). Prout also proposed a consequent alkalinization of the blood during acid secretion. Later on, Schwarz (1910) enforced the importance of back-diffusing acid in the development of ulcers with his renowned dictum — "*No acid…No ulcer*".

Glover, in 1800, implicated the mucus layer as a physical barrier to luminal acid and bile. This concept was emphasized by Harley in the 1860's and led to an elevated interest in the mucus layer as a barrier to digestive enzymes in the mid-20th century. This notion of the impermeability of the mucus layer and mucosa was however proposed by Bernard already 1855, although it could not be convincingly shown at that time. These results, among others, lay the basis for Hollander's concept of a "two-component self-generating mucous barrier" (Hollander 1954). The barrier comprises an outer layer of viscous gel "*mucus*" and an epithelial cell layer immediately beneath. The dual character of this barrier was also suggested for the duodenum. Hollander envisaged the secretion of two different types of mucus — a highly viscous secretion from the surface cells and a mucoid fluid from the neck chief cells. The protective quality of the mucus layer was ascribed to its high viscosity protecting the mucosa from mechanical shear injury. Pavlov (1898) first depicted that an alkaline mucus gel layer neutralized luminal acid. He later (1910) suggested that the flow action of the mucus layer and a liquefication of the mucus gel during acidification was a mechanism to flush away luminal pepsin and noxious agents. However, a role for the mucus layer as a barrier to diffusion was suggested in the 1950's (Heatley, 1959). Heatly proposed that the mucus layer provided an unstirred layer in which back-diffusing acid is neutralized by bicarbonate secreted from the mucosa, thereby creating a standing pH–gradient from near neutral at the mucosal surface to acidic in the lumen. Hollander also recognized the extraordinary rapidity

by which the epithelial cell layer could restore its continuity after injury, a phenomenon now known as restitution or “rapid repair” (Eastwood, 1991). This property can be ascribed to the continuous mitotic activity in stem cells, which supply new migrating cells and maintain a fairly rapid turnover of the epithelial cells lining the gut.

Over the last three decades studies of the gastrointestinal protective mechanisms have intensified. Today, it is generally accepted that a laminar defense system of three major layers consisting of pre–epithelial (mucus–bicarbonate), epithelial (apical cell membrane, intercellular tight junctions, plasma membrane exchangers and restitution), and sub–epithelial (blood flow, mucosal nerves and immune system) components protect the mucosa from injury (Fig. 1).

In 1982, after a hundred years of searching, the microbiological discovery of the century was made; the bacterium *Helicobacter pylori* was direly implicated in the development of gastroduodenal inflammation and ulcers (Kidd and Modlin, 1998). An intense research has followed but the pathophysiology of *H. pylori’s* contribution to gastroduodenal injury is still not fully understood.

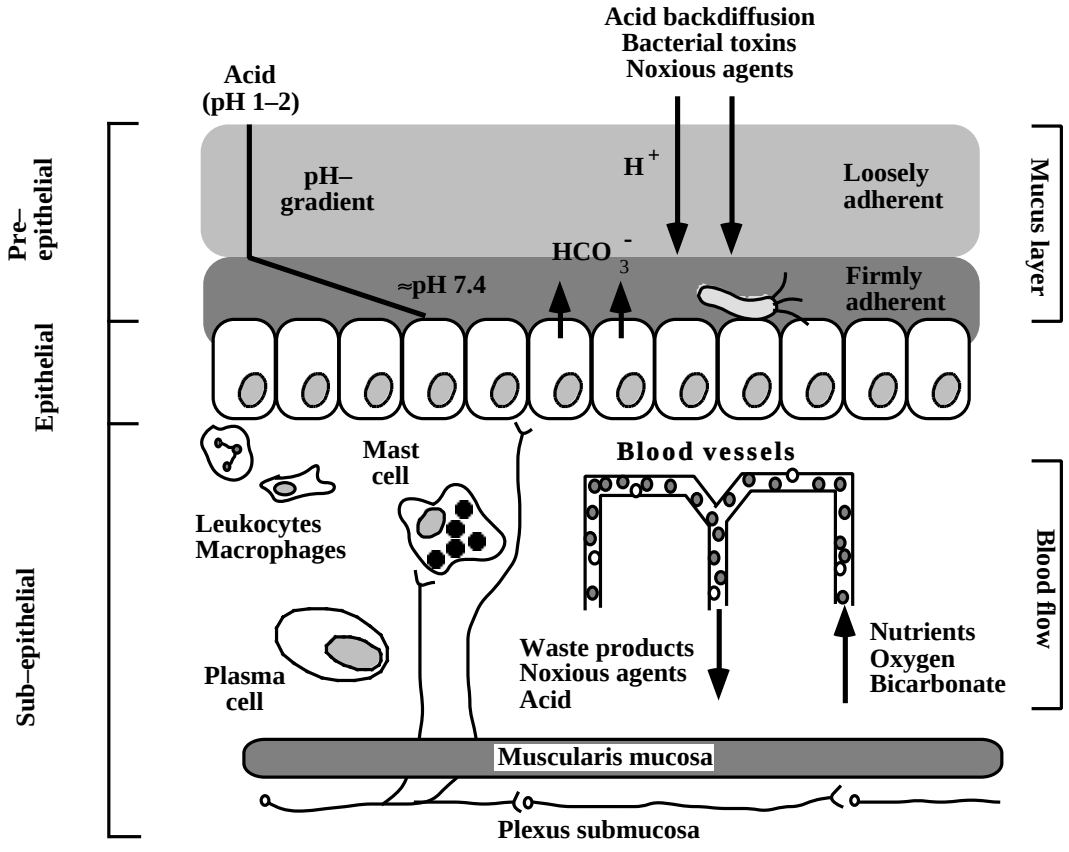

Figure 1. *Schematic diagram of the three levels of mucosal defense. NB: The figure is not drawn to scale.*

The gastrointestinal tract

The mouth is the front porch and the stomach the hallway of the gut with the pylorus functioning as a gateway, restricting and regulating the passage of the gastric luminal contents into the intestine. After passage the luminal contents must pass through the entire intestine

with only the thin mucosa as a protective barrier. The main function of the stomach is to further facilitate the enzymatic digestion of food, following the actions of the salivary enzymes, and to continue the mechanical degradation into a semi–liquid chyme. In the duodenum and proximal jejunum of the small intestine the enzymatic digestive processes are furthered by enzymes in the pancreatic and bilary secretes. Throughout the small intestine including the distal jejunum and ileum, nutrients and fluid are absorbed through active and passive processes. The concentration of the residual products with respect to fluid content is regulated in the proximal and mid colon. The distal colon and rectum function mainly as a reservoir until the propulsive forces of the intestine and the conscious will of the individual depict that the faeces can be excreted.

Anatomy in brief

The major anatomic structures of the gastrointestinal tract are the mucosa, submucosa, muscularis and serosa (Ito, 1987, Madara and Trier, 1987). The mucosa constitutes an epithelial lining overlying the lamina propria composed of supporting cells, small blood vessels, lymphatics, nerve fibers and immune cells, including mast cells, leukocytes and macrophages. The lamina propria rests on a thin muscle layer, the muscularis mucosae, arranged as two or three sublayers. The submucosa consists of relatively dense connective tissue and harbors part of the enteric nervous network, the submucosal plexus. Traversing the submucosa are larger blood vessels and lymphatics supplying or draining their equivalents in the lamina propria. More peripherally, the muscularis is made up of two or three sublayers of smooth muscle. Between these layers resides the second part of the enteric nervous network, the mesenteric plexus. Enfolding the inner regions is the serosa; a thin layer of loose connective tissue covered by flattened squamos cells, the mesothelium.

The rat stomach can be divided into three distinct regions: the proximal forestomach, the oxyntic corpus region and the distal antrum (reviewed in Helander, 1981, and Ito, 1987). Contrary to the case in man, the rat forestomach is not a part of the acid-producing oxyntic region called the fundus. The rat forestomach is lined with a layer of stratified squamos cells, has a non–glandular mucosa and mainly functions as a reservoir. Conversely, the corpus and antrum have a glandular mucosa with down-growths of cylindrical gastric glands. Gastric glands of the corpus are lined with parietal cells that secrete acid, chief cells that secrete the proteolytic enzyme pepsinogen, and a few enteroendocrine cells, which secrete locally acting humoral factors. In the antral mucosa there are endocrine cells involved in the regulation of acid secretion; G–cells produce gastrin that stimulates acid secretion, whereas acid sensitive D–cells produce somatostatin that inhibits acid secretion. The glands open into the bottom of funnel shaped gastric pits with the neck region containing the mucous neck cells, which secrete mucus. The surface epithelial cells of the corpus and the antrum secrete mucus that contributes to that from the mucous neck cells in forming a continuous protective gel blanket covering the mucosal surface.

In the small intestine, the mucosa is thrown into folds, villi, to increase the absorptive area. The epithelial enterocytes are the main absorptive cells of the villi and each cell forms several thousand small luminal protrusions, microvilli, which increase the absorptive area even more. Between the villi the mucosa bulges down into the cylindrical crypts of Lierberkühn, which are lined with cells specialized in fluid, chloride and bicarbonate secretion. Both the villi and the crypts also have endocrine cells important in regulating stomach, gallbladder and pancreatic function. A protective mucus layer is secreted by numerous goblet cells distributed in the epithelium. The epithelial volume density of goblet cells is fairly consistent in the crypts, but in the villus epithelium it increases aborally from the duodenum to the distal ileum

(Moe, 1955, Specian and Oliver, 1991). Brunner's glands are located in the first few centimeters of the duodenum and are particularly enriched in mucus and bicarbonate secreting cells (Madara and Trier, 1987). Secretion of fluid and mucus are important defense mechanisms, which serve to dilute and wash away noxious substances from the epithelial surface.

The colonic mucosa, similar to the small intestine, has crypts of Lierberkühn but lacks villi. The epithelium is very richly interspersed with mucus secreting goblet cells with the volume density of the cells increasing in the caecum to rectum direction (Specian and Oliver, 1991). A mucus gel layer covers the colonic surface and houses the major part of our intestinal microbial flora (Simon and Gorbach, 1987).

The crypts of all segments also hold the stem cells from which all epithelial cells are derived (Lipkin, 1987). The stem cells divide continuously replacing the various epithelial cells regularly with turnover times of a few days or more, depending on location and function. Stem cells are a prerequisite and of utmost importance for the restitution of the epithelial cell layer following injury ("*rapid repair*") (Silen, 1987, Eastwood, 1991).

The gastrointestinal mucus gel layer

An adherent mucus gel layer covers the surface of the gastrointestinal mucosa from the stomach to the colon, and constitutes the site of the first line of defense against luminal aggressors (Allen and Garner, 1980). The mucus gel layer acts as a diffusion barrier against noxious agents, entraps microorganisms, interacts with the immune surveillance system and by acting as a lubricant enhances the propulsion of chyme down the gastrointestinal canal (Silen, 1987, Allen *et al.,* 1993). For the mucus layer to provide this effective barrier it ought to be continuous, which has been shown for the duodenum (Sababi *et al.,* 1995) and stomach (Schade *et al.,* 1994, Jordan *et al.,* 1998). However, the question of its continuity and thickness has been debated over the years since different techniques and experimental models have yielded contradicting results. Futhermore, the role of the mucus layer varies in the different segments of the gastrointestinal tract and thus, the biophysical properties and thickness of the mucus gel may vary accordingly. Normally, the thickness of the adherent mucus layer is a balance between its secretion rate and its erosion through enzymatic degradation by luminal enzymes and mechanical shear. Consequently, the mucus layer has a fairly rapid turnover time. The protective quality of the mucus gel layer is dependent on its stability and physical and chemical properties. In keeping with this, it is conceivable that disrupting the balance between degradation and renewal rates or its properties would jeopardize the integrity of the underlying mucosa. Indeed, this has been seen in peptic ulcer disease and inflammatory bowel disease (Copeman and Allen, 1994, Pullan *et al.,* 1994, Allen *et al.,* 1998, Schultsz *et al.,* 1999). An important consideration in this reasoning is that the thickness of the mucus layer and its regulation (Tabata *et al.,* 1992) and also mucin gene expression (Buisine *et al.,* 1998) may vary with age. In addition, the mucin gene expression and hence the quality of the mucus produced, may differ for example during *H. pylori* infection (Byrd *et al.,* 1997) and in neoplastic gastrointestinal tissue (Lesuffleur *et al.,* 1994).

Mucus glycoproteins

Mucus is a cohesive mixture of approximately 95% water, 5% mucin glycoprotein molecules, salts, immunoglobulins, cellular and serum macromolecules, and trefoil peptides (Allen *et al.,* 1993, Wong *et al.,* 1999). Mucin molecules are high molecular weight glycoproteins (500–30 000 kDa) with a protein backbone, parts of which are glycolated with non–glycolated "*naked*" stretches in between (Allen and Garner, 1980, Neutra and Forstner, 1987, Specian and Oliver, 1991, Allen and Pearson, 1993). The gel forming properties of the mucins can be attributed to the ability of

the oligosaccharide side chains to stretch out and hydrate, and to polymerize and form disulfide bonds between cystein amino acids in the naked regions of their backbones (Allen and Garner, 1980, Gum jr., 1995, Allen *et al.,* 1998). The ability to form non-covalent bonds between mucins has also been suggested as an important gelation mechanism (Bansil *et al.,* 1995). In keeping with this, the mucus gel consists of a large polymeric mesh or a disperse network, with strands of mucins interconnected by disulfide bonds. An interesting finding is what has been called a "link protein" of ≈118 kDa, which interconnects several gastrointestinal mucins and is probably cleaved off from the original protein core of parent mucins (Roberton *et al.,* 1989, Allen and Pearson, 1993a). An additional function of the oligosaccharide side chains is to protect the protein core from proteolytic digestion (Allen and Garner, 1980, Allen and Pearson, 1993). However, the different mucin types may have different amounts of tandem repeat regions in the protein core, containing O–glycosylation sites, which would affect the physical and/or biological properties of the mucin (Gum jr., 1995).

To date, eleven distinct mucin genes have been discovered (MUC1–4, MUC5AC, MUC5B, MUC6–8, MUC11 and MUC12). The localization of the mucin gene products varies throughout the gastrointestinal tract and the other mucus secreting systems in the body (Lesuffleur *et al.,* 1994, Van Klinken *et al.,* 1995, Gendler and Spicer, 1995, Williams *et al.,* 1999). Mucins exist as a heterogeneous population, but in the stomach MUC5AC and MUC6 are generally expressed in the surface epithelial cells and mucous neck cells, respectively, and are the major mucin products (Ho *et al.,* 1995, Porchet *et al.,* 1995). In the small intestine and colon MUC2 is the dominating mucin product (≈80% of mucins) secreted from goblet cells (Tytgat *et al.,* 1994, Karlsson *et al.,* 1996, Allen *et al.,* 1998, Van Klinken *et al.,* 1999), although MUC3 has also been found (Van Klinken *et al.,* 1995, Gum jr., 1995).

Another interesting class of secretory proteins are the trefoil peptides, which are produced and secreted together with the mucins and are present in fairly high concentrations in the mucus gel layer and in the mucosa (Wong *et al.,* 1999, Newton *et al.,* 2000). Their functions are not yet clear, but they are thought to be intimately associated with mucus and improve its resistance to noxious agents. They are upregulated at all sites of injury and have been implicated in promoting cell migration and stimulating the repair process.

Mucus secretion

Surface mucus cells show three modes of mucus discharge: single granule exocytosis, apical expulsion or compound exocytosis, and cell exfoliation (Zalewsky and Moody, 1979, Specian and Oliver, 1991, Forstner and Forstner, 1994, Forstner, 1995). Baseline secretion is probably maintained by unstimulated release of single granules by fusion of the peripheral secretory granules with the plasma membrane. In one pilot study the spontaneous baseline secretion amounted to 1–2 granules per cell and 5 min period in the rabbit (Matsushita *et al.,* 1998). Apical expulsion or compound exocytosis was observed as a rapid burst of exocytosis upon mechanical stimulation. This accelerated exocytosis was characterized by the opening of fusion pores in a sequential manner between previously fused and adjacent granules resulting in emptying and cavitation of the apical granule storage area. Apical expulsion is an extreme process involving the loss of cytoplasm and excess granule membrane and can be completed within 30 min. However, the intestinal goblet cells recover fairly quickly and are refilled in 60–120 min, with the longer recovery period for goblet cells in the colon. Cell exfoliation occasionally occurred even in the absence of a secretagogue, characterized by the migration of the cells into the lumen — could this reflect normal cell turnover?

Regulation of mucus secretion has been coupled to neural, hormonal and paracrine effects (Forstner and Forstner, 1994, Plaisancié *et al.,* 1998). Mucus secretion may be increased by NO (Brown *et al.,* 1993, Sababi *et al.,* 1995), PGE_2 (Sababi *et al.,* 1995, Plaisancié *et al.,* 1998), histamine (Neutra *et al.,* 1982, Halm and Troutman Halm, 2000) and substance P (Castagliuolo *et al.,* 1996), etc. A note of caution, however, as mucus producing cells in different sites may be and probably are regulated differently (Neutra *et al.,* 1982, Ichikawa *et al.,* 1998, Halm and Troutman Halm, 2000).

Mucus function

The protective functions of the mucus gel layer have mostly been studied in the stomach and duodenum. It is permeable to ions and smaller molecules, but restricts diffusion of macromolecules including the *H. pylori* cytotoxin VacA and cholera toxin (Flemström *et al.,* 1999) and pepsin (Allen *et al.,* 1993). However, it impedes the diffusion of acid from the lumen (Williams and Turnberg, 1980, Vadgama and Alberti, 1983) and provides a stable unstirred layer for neutralization of the acid by mucosal bicarbonate secretion (Engel *et al.,* 1984, Kiviluoto *et al.,* 1993, Hogan *et al.,* 1994, Livingston *et al.,* 1995, Engel *et al.,* 1995). Consequently, a pH gradient is formed with a near neutral pH close to the epithelium and an acid pH luminally (Heatley,1959, Williams and Turnberg, 1981, Flemström and Kivilaakso, 1983, Takeuchi *et al.,* 1983, Paimela *et al.,* 1990, Schade *et al.,* 1994). This concept has lately been challenged in a study that described an acidic (pH4.2) juxtamucosal environment during luminal pH5 (Chu *et al.,* 1999). Interestingly, a recent discovery suggests that secreted acid and pepsinogen is transported through distinct channels in the mucus layer, thereby preventing juxtamucosal acidification and access of pepsin to the mucosal surface (Holm and Flemström, 1990, Johansson *et al.,* 2000). The driving force for the formation of these channels has been proposed to be the high intraglandular pressure previously measured in the lumen of the gastric gland (Holm *et al.,* 1992, Synnerstad *et al.,* 1997, Synnerstad and Holm, 1998).

Much less is known about the functions of the mucus gel layer in the remainder of the gut. A common function is that of lubrication to minimize shear injury and ease the propulsion of luminal contents. The mucus gel in the ileum and colon has been suggested to harbor a major part of the intestinal flora (Simon and Gorbach, 1987). In other parts of the gut the mucus gel has receptors for a wide range of microbial adhesins and contains high levels of secreted IgA. These resources are used to immobilize microorganisms and protect the mucosa against adherence and invasion of pathogens, e.g. *Campylobacter jejuni, Salmonella typimurium* and *H. pylori* (Slomiany and Slomiany, 1991, Forstner and Forstner, 1994).

In case of mucosal injury the process of rapid repair or restitution commences to rapidly reinforce the integrity of the mucosa (Eastwood, 1991). During this healing process the mucins together with fibrin and necrotic cells, form a thick gelatinous "*mucoid cap*" under which re–epithelialisation can proceed in a near neutral milieu (Ito and Lacy, 1985, Allen *et al.,* 1987, Wallace and McKnight, 1990).

Properties of the mucus layer

Several different techniques have been employed to study and characterize the mucus gel layer. These include *in vitro* studies of unfixed sections using an inverted microscope with calibrated graticula (Kerss *et al.,* 1982, Sandzén *et al.,* 1988, Rubinstein and Tirosh, 1994, Pullan *et al.,* 1994) or studies of inverted mucosa with a slit lamp and pachymeter (Bickel and Kauffman, 1981) or microelectrode (Takeuchi *et al.,* 1983). Several histological techniques have also been used (Szentkuti and Lorenz, 1995, Matsuo *et al.,* 1997, Jordan *et al.,* 1998). The studies above have usually only covered limited segments of the gastrointestinal tract and the results have varied immensely. A major reason has been the loss of mucus during the handling procedure and the

dehydration/shrinkage of the mucus gel (Jordan *et al.,* 1998). A few *in vivo* studies have been performed with generally thicker mucus thickness values (Kaunitz *et al.,* 1993, Schade *et al.,* 1994, Sababi *et al.,* 1995), except for a recent study using the confocal microscope (Chu *et al.,* 1999). To date no *in vivo* study has been performed to study the thickness and properties of the mucus gel layer throughout the gastrointestinal tract and without distortion of the gel layer.

Three phases of the gastroduodenal mucus layer have been proposed: presecreted mucus stored in the epithelial cells, a firmly adherent mucus gel layer and mobile (largely soluble) luminal mucus (Allen and Carroll, 1985, Allen *et al.,* 1993). However, recent *in vitro* results using histological techniques suggest that the adherent mucus layer may be made up of two or more physically distinct layers (Ota *et al.,* 1992, Matsuo *et al.,* 1997) that are possibly made up of laminar arrangements of gels with different mucin content (Ota *et al.,* 1992, Ishihara and Hotta, 1993, Ho *et al.,* 2000).

Lipids have been suggested to be involved in building up and strengthning the mucus layer. A surfactant-like layer of surface-active phospholipids was described to cover the mucosal surface (Hills, 1992, Mauch *et al.,* 1993). This hydrophobic barrier has also been suggested to coat the mucus layer with high values in the stomach and colon, and has been deemed necessary for the ability of the mucus layer to resist luminal acid (Lichtenberger, 1995). This hypothesis of a surface-active layer is still a matter of debate and other results suggest that the phospholipids actually are an integral part of the mucus layer (Slomiany and Slomiany, 1991).

The mucus layer in H. pylori *infection*

The weakening of the protective mucus gel layer by *H. pylori* is yet controversial. Numerous bacteria reside spread out in the mucus gel and attached to the mucosa releasing various mucolytic and proteolytic enzymes. The mode of action can thus be a direct effect on the gel or effects on the synthesis and/or release of mucins from the mucus producing cells. Recently, *H. pylori* has been seen to co–localize with MUC5AC suggesting that this mucin, secreted by the surface epithelial cells, is involved in the adhesion of the bacteria to the mucosa (Van den Brink *et al.,* 2000).

H. pylori may affect the structure of the mucus gel layer (Younan *et al.,* 1982, Slomiany *et al.,* 1987, Sarosiek *et al.,* 1988, Sidebotham *et al.,* 1991). These structural changes in the mucus layer were not associated with a decrease in mucus thickness (Allen *et al.,* 1997) and may not necessarily imply a collapse of the mucus barrier (Newton *et al.,* 1998). However, studies of biopsies from *H. pylori* infected patients have shown a decrease in mucus thickness (Sarosiek *et al.,* 1991). In addition, the hydrophobicity of the mucus layer was reduced in biopsies from *H. pylori* infected patients (Spychal *et al.,* 1990, Mauch *et al.,* 1995, Asante *et al.,* 1997). To complicate matters further, Markesich *et al.* (1995), have found that the mucus gel viscosity increases during *H. pylori* infection and Worku *et al.* (1999), have recently shown that a high mucus viscosity impairs the motility of the bacteria — Is this a possible defense mechanism? A different angle is the effect on mucus secretion. *H. pylori* has been observed to attenuate both basal and stimulated mucus secretion in cultured cells (Micots *et al.,* 1993, Takahashi *et al.,* 1998), and reduce mucin synthesis (Byrd *et al.,* 2000). A possibility is that *H. pylori* may prevent the fusion of the secretory granules with the apical surface membrane (Micots *et al.,* 1993).

The breakdown of the mucus structure is probably a result of the mucolytic effects of *H. pylori* enzymes (Sarosiek*et al.,* 1991a). However, an increased pepsin 1 secretion has been seen in peptic ulcer disease, which may contribute to mucus breakdown (Copeman and Allen, 1994). Byrd *et al.* (1997), showing an aberrant expression of the gland-type mucin (MUC6) in the

surface epithelial cells, presented a clue to the detrimental effects of the bacteria on the adherent mucus layer. Indeed, *H. pylori* infection disrupts the suggested laminated structure of the mucus gel layer, which is restored after eradication (Shimizu *et al.,* 1996).

The very divergent results may in part be due to differences in the stage of the infection but could also be the result of strain variation. Again, these studies including mucus thickness measurements have been performed *in vitro* on biopsies from patients with a chronic infection. What are the early effects on the mucus layer and what are chronic effects?

Gastric blood flow

It is now generally accepted that an adequate mucosal blood flow is a prerequisite for maintaining mucosal integrity. The protective mechanisms of the mucosal blood flow have been addressed from several aspects. It has been shown to be necessary for diluting and flushing out back-diffusing acid (McGreevy and Moody, 1977, Cheung *et al.,* 1977) and cellular waste products (Holzer *et al.,* 1994). Furthermore, the blood flow is a source of bicarbonate for the surface epithelial cells which is necessary for intramural neutralization of acid and maintenance of the pH–gradient in the overlying mucus layer (Kivilaakso, 1981, Starlinger, 1988). Adequate oxygen and nutrient delivery is also vital for maintenance of the metabolic process and the process of rapid repair (Guth and Leung, 1987, Guttu *et al.,* 1994).

Blood vessel arrangement

The anatomic arrangement of the sub–epithelial capillaries is ingenuously designed to transport bicarbonate from the parietal cells in the glands to the surface epithelial cells (Silen, 1987). This bicarbonate transport is increased during acid secretion, the so-called "*alkaline tide*". The supplying arteries enter the external muscle layer and give off branches that supply the superficial and deep muscle layers (Gannon *et al.,* 1982, Gannon *et al.,* 1984, Guth and Leung 1987). In the submucosa the arteries form arcade networks by anastomosing amongst themselves and forming successively smaller arcades. At the base of the mucosa the arterioles divide into fenestrated capillaries, sent up perpendicular to the mucosa, engulfing the glands and forming a sub–epithelial honeycomb network immediately beneath the luminal surface. Several laterally oriented connections occur between the capillaries in the mucosa. The capillaries drain into large collecting venules at the sub–epithelial level that in turn traverse the mucosa and join together at the base of the mucosa to form the venous submucosal plexus. These veins drain into larger veins that follow the course of the supplying arteries. This vascular arrangement ensures a unidirectional blood flow in the mucosa and the maintenance of an adequate acid–base balance (Silen, 1987, Wallace and Granger, 1996). In general, the blood vessels at the basal part of the mucosa and in the submucosa are densely innervated, while the vessels in the superficial mucosa are almost devoid of innervation (Guth and Ballard, 1981, Keast *et al.,* 1985). Thus, autonomic regulation of the mucosal perfusion is mainly mediated via the arterioles and veins in the submucosal region.

Piasecki (1992), reported the occurrence of "mucosal end–arteries of extramural origin" in areas, which co–localize with the commonly recognized ulcer sites in the lesser curvature and proximal duodenum. These arteries do not connect with the submucosal plexus and therefore, the supplied mucosal regions are more prone to be hypoperfused due to vascular obstruction during focal spasm of the external muscle layers. These structures are not found in all individuals, which was suggested to be the reason why certain people were more susceptible to injury by stress, acid and *Helicobacter pylori* infection. Other studies have found a gradient with lower blood flow in the pre–pyloric region and proximal duodenum compared to the

proximal region of the stomach (Allen *et al.,* 1988) and lower blood flow in the lesser curvature compared to the greater curvature (Lunde and Kvernebo, 1988).

Blood flow and mucosal injury

The importance of mucosal blood flow for mucosal protection is based on studies of the effects of a luminal challenge with an aggressor during the induction of a decreased or increased blood flow (reviewed in Guth and Leung, 1987 and Wallace and Granger, 1996). Numerous studies point at an ischemic pathogenesis in the development of acute and chronic ulcers. Topical acid is a frequently used aggressor with concomitant induction of ischemia by drugs or by mechanical obstruction. Acid, at a concentration of 150mM (HCl), did not cause damage in the normotensive rat whereas concentrations of 50mM caused erosions in the ischemic mucosa (Mersereau and Hinchey, 1973). Furthermore, studies employing other barrier breakers as well, e.g. ethanol and bile with similar results, have offered further credence to the hypothesis (Whittle, 1977, Cheung and Chang, 1977, Leung *et al.,* 1985, Pihan *et al.,* 1986, Guth, 1986, Szabo *et al.,* 1986). The study by Leung *et al.*, suggested a 40-% reduction in blood flow and perfusion pressure as a threshold for increased acid induced injury. Ritchie jr. (1975) demonstrated that the ratio between the amount of back diffusing hydrogen ions and the mucosal blood flow was crucial to the severity of the ulceration. Hence, a hyperemic response to luminal irritants seems to be an essential component of the gastric defense system, since its prevention leads to the development of hemorrhagic necrosis.

In concurrence, several investigators show that a hyperemic response protects the mucosa from pending injury and have performed studies of the mechanisms behind this effect. An increased release of prostaglandins (Whittle and Vane, 1987) and of calcitonin gene related peptide (CGRP) from local nerves (Guth, 1992, Li *et al.,* 1992) has been seen to increase blood flow in response to backdiffusing acid. Capsaicin-sensitive sensory neurons seem to signal for and mediate the hyperemic response to luminal acid (Holzer *et al.,* 1991, Holzer *et al.,* 1991a). The hyperemic effect of CGRP is probably mediated by the vasodilator action of nitric oxide (NO) formed in the endothelial cells of the vessel wall (Whittle *et al.,* 1990, Holzer *et al.,* 1994). Another interesting finding is that luminal acid, although a potent stimulator of mucosal blood flow, may further reduce blood flow in the presence of ischemia (Mersereau *et al.,* 1973, Stein *et al.,* 1989). Thus, once blood flow has been reduced, even if in a focal manner, it can be compromised further by luminal acid with consequent ulceration (Allen *et al.,* 1993). Recent studies have shown that the neurotransmitter substance P (SP) is co–released with CGRP and may impair the CGRP-mediated hyperemia by a mast cell-dependent mechanism (Grønbech and Lacy, 1994, Rydning *et al.,* 1999). Indeed, SP aggravates ethanol-induced damage to the gastric mucosa by the stimulation of mast cells (Karmeli *et al.,* 1991). In addition, an increased level of SP has been found in the duodenal mucosa of duodenal ulcer patients (Domschke *et al.,* 1985) and SP may mediate the inflammatory response to luminal toxin from *Clostridium difficile* (Pothoulakis *et al.,* 1994Mantyh *et al.,* 1996).

It is still debatable as to the causal relationship between a reduction in blood flow and mucosal injury. An obvious question is if the reduction in blood flow is the cause or consequence of mucosal damage. A reduction in mucosal blood flow has been seen as an early event of mucosal injury (Guth, 1986, Pihan *et al.,* 1986, Szabo *et al.,* 1986, Szabo, 1987, Allen *et al* 1993). *H. pylori* has been found to lower mucosal blood flow *in vivo* in patients with a chronic infection (Lunde and Kvernebo, 1988). However, it is still unknown what happens in the early stage of infection. Vasoconstriction and increased vascular permeability with subsequent leakage of plasma (Szabo *et al.,* 1986) may reduce blood flow. An early constriction of mucosal vessels has been suggested to be the result of an abnormal motility pattern, which may cause

an infarction-like mechanism to induce mucosal ulceration (Piasecki 1992). Damage to the surface epithelial cells could upregulate vascular adhesion molecules with subsequent recruitment and clogging of the microvessels by neutrophils and platelets (Guth, 1992, Smith *et al.,* 1987, Panés and Granger, 1998). The neutrophils in turn secrete tissue-damaging factors in the vessels and in the tissue on extravasation.

Mast cells

Ehrlich, in the 1870's, made a detailed characterization of the metachromatic connective tissue cells called "mastzellen" previously detected by von Recklinghausen (1863) (Reviewed in Selye, 1965). Some years later, Unna (1894) discovered the clinical relevance of mast cells in studies of cutaneous lesions in urticaria pigmentosa where the mast cell population was increased. Cazal (1942) made the first suggestion of a relationship between mast cells and histamine release and Benditt (1955) found that mast cells release serotonin. The mast cell was found to degranulate upon contact with irritants and to be related to anaphylactoid inflammation.

Distribution

Today, mast cells are known to exist in most tissues, especially those that come into contact with the external environment, such as the skin, airways and gastrointestinal tract. Mast cells form a heterogeneous group that has been subdivided in the gastrointestinal tract into mucosal mast cells and connective tissue mast cells. These two cell types vary in their mediator content and in their degranulatory response (Barrett and Metcalfe, 1984, Kagnoff, 1987). Mucosal mast cells are found in the lamina propria from the base of the glands to the muscularis mucosa and between gastric pits near the lumen (Grønbech and Lacy, 1994). Connective tissue mast cells, on the other hand, are concentrated to the submucosa and serosa with particular abundance close to muscularis mucosa.

The gastrointestinal tract is relatively rich in mast cells, containing approximately 20 000 mast cells per mm^3 (Barrett and Metcalfe, 1984, Kagnoff, 1987). Mast cells, together with macrophages, residing in the lamina propria act as "alarm cells". They respond to foreign matter by releasing soluble mediators and cytokines, which initiate an inflammatory response (Wallace, 1996). The involvement of mucosal mast cells in a variety of disease states associated with chronic inflammation has emerged. Some of these are *H. pylori* gastritis and peptic ulcer disease (Plebani *et al.,* 1994, Nakajima *et al.,* 1997, Yamamoto *et al.,* 1999), *C. difficile* infection (Pothoulakis *et al.,* 1993, Castagliuolo *et al.,* 1994, Wershil *et al.,* 1998) and nematode infection (Perdue *et al.,* 1990). Mast cells have also been implicated in ethanol and acid injury to the gastric mucosa (Karmeli *et al.,* 1991, Rydning *et al.,* 1999).

Regulation

Several studies suggest communication between mucosal mast cells and nerves. However, does this represent a functional unit such that released neurotransmitters activate mast cells or vice versa? Mucosal mast cells are closely apposed to the enteric nerves (Stead *et al.,* 1989). In the intestine the proportion of mast cells in direct contact with peptidergic nerves was between 47% and 78% (Stead *et al.,* 1989). A number of mast cells are also found in close proximity to nerves (Stead *et al.,* 1987). Both SP and CGRP have been localized in these nerves (Stead *et al.,* 1987, Maggi, 1997). SP containing nerves are one of the most predominant nerve types in the mucosa (Keast *et al.,* 1985, Green and Dockray, 1988) that are activated by excessive distension or irritative stimuli (Costa *et al.,* 1986). Changes in epithelial function and properties in connection with mast cell degranulation (Stead *et al.,* 1987, Perdue *et al.,* 1990, Crowe and Perdue,

1992, Crowe and Perdue, 1992a, Wallace and Granger, 1996) provide further evidence for an interaction between mucosal nerves, mast cells and the epithelium to promote antigen-dependent, functional physiological changes. Mast cell reactivity is suppressed by prostaglandins (Hogaboam *et al.,* 1993, Wallace and Granger, 1996) and NO (Kubes *et al.,* 1993, Kanwar *et al.,* 1994, Alican and Kubes, 1996, Wallace and Granger, 1996). Indeed, mast cells may release a NO-like factor that can regulate its function in an autocrine fashion (Mansini *et al.,* 1991). It should be kept in mind that eosinophils and plasma cells might also be similarly innervated in the mucosa (Stead *et al.,* 1987, Stead, 1992).

Mediators

Mast cell degranulation per se involves the release of several preformed mediators such as histamine, serotonin and cytokines and newly formed mediators such as platelet activating factor (PAF) and leukotrienes (Barrett and Metcalfe, 1984, Crowe and Perdue, 1992a, Kubes and Granger, 1996, Wallace and Granger, 1996). These mediators have effects on the microvasculature, mucus secretion, epithelium and recruitment of inflammatory cells. Mast cell mediated effects have been successfully attenuated with the mast cell stabilizer ketotifen (Craps and Ney, 1984) and lidocaine (Castagliuolo *et al.,* 1994).

The platelet activating factor

PAF is an endogenous alkyl phospholipid mediator, formed from the breakdown of membrane phospholipids, that has diverse and potent effects on many different cell types. PAF is released from a number of inflammatory cells including mast cells, leukocytes and platelets found in the mucosa, endothelial cells (Bonavida and Mencia–Huerta, 1994) and from *H. pylori* (Denizot *et al.,* 1990). Many of the cells or tissues that generate PAF are also its targets. PAF has been implicated in a number of clinical states such as acute inflammation, ischemia, arterial thrombosis, endotoxic shock and acute allergic diseases (Braquet *et al.,* 1987, Koltai *et al.,* 1992, Bonavida and Mencia–Huerta, 1994). The unifying theme being microvascular failure including early leukocyte recruitment.

PAF pathophysiology

PAF has extremely potent ulcerogenic actions in the stomach causing extensive damage extending throughout the mucosa (Rosam *et al.,* 1986). These actions are in part due to its ability to recruit and activate leukocytes (Kubes *et al.,* 1990, Wood *et al.,* 1992, Arndt *et al.,* 1993, Gaboury *et al.,* 1995, Kubes and Granger, 1996) and also to its direct vasoconstrictor action and increased muscular contractions (Tepperman and Jacobson, 1994). PAF is reported to increase gastric vasocongestion and vasoconstriction (Rosam *et al.,* 1986, Whittle *et al.,* 1986, Wallace *et al.,* 1987, Wood *et al.,* 1992, King *et al.,* 1995, Kubes and Granger, 1996). In addition, PAF increases mucosal vascular permeability, which by increasing blood viscosity may further reduce blood flow (Wallace *et al.,* 1987). It also seems to mediate the gastrointestinal damage associated with endotoxic shock (Wallace *et al.,* 1987, Koltai *et al.,* 1992), as bacterial endotoxins cause the generation of PAF in the blood and tissues of animals (Bonavida and Mencia–Huerta, 1994). Interestingly, a recent study has found an increased mucosal production of PAF in *H. pylori* infected children (Hüseyinov *et al.,* 1999).

The actions of PAF seem to vary with tissue concentrations since low doses induce vasodilation and high doses induce vasoconstriction in the arterioles of the gastrointestinal tract (King *et al.,* 1995). In addition, macroscopically assessed damage, vasocongestion and necrosis, was dose-dependent, and low doses without systemic hypotensive actions induced gastric damage in the presence of 20% ethanol (Esplugues and Whittle, 1988). In concert with this, low doses of PAF regulate interleukin–1 (IL–1) release from macrophages and IL–1 in turn

inhibits PAF release from mast cells by a NO-dependent mechanism (Bonavida and Mencia–Huerta, 1994). PAF may therefore have a role in the fine-tuning of the early inflammatory response.

PAF receptor antagonists have been successfully used to inhibit the development of and reverse many aspects of the PAF-induced pathophysiology (Wallace *et al.*, 1987, Braquet *et al.*, 1987, Kubes *et al.*, 1990, Arndt *et al.*, 1993, Bar–Natan *et al.*, 1995, Gaboury *et al.*, 1995, Laniyonu *et al.*, 1997). Of particular interest in this thesis are the results of using PAF-receptor antagonists to counteract the effects of a water extract from *H. pylori*. On superfusion of the mesentery, Kurose *et al.* (1994), found an early albumin leakage at 10 min and a late albumin leakage after 30 min, accompanied by increased leukocyte adhesion and emigration. The PAF-receptor antagonist WEB2086, effectively attenuated the early increase in vascular leakage and late leukocyte emigration. Leukocyte adhesion and the late phase vascular leakage were unaffected as has been shown earlier (Yoshida *et al.*, 1993). The same result was obtained by mast cell stabilization with ketotifen. Their conclusion was that the early phase was mast cell and PAF dependent while the later phase was dependent on leukocyte endothelial cell interactions and platelet–leukocyte aggregates. Contradicting results were obtained in a recent study in which the PAF-receptor antagonist, hexanolamine–PAF, could not attenuate vascular leakage, but did inhibit platelet aggregation on luminal challenge with the extract in the stomach (Kalia *et al.*, 2000). However, ketotifen inhibited the transient vascular albumin leakage peaking at 5 min, but had no effect on platelet aggregation. Leukocytes were neither observed to adhere and emigrate in blood vessels nor found in the platelet aggregates. This later study is in conjuncture with an earlier study, where intravenous infusion of PAF in the stomach caused congestion and stasis of the mucosal capillaries but did not induce any leakage of plasma proteins in the mucosa (Whittle *et al.*, 1986). On the other hand, an intradermal injection of PAF in the rat was followed by vascular leakage, edema, vascular lesion and platelet thrombosis (Braquet *et al.*, 1987). PAF activation of platelets in the rat is thought to be through an indirect mechanism (Braquet *et al.*, 1987).

Of note is that the various experiments administer HPE or PAF differently and that the studies concern different areas, mesentery contra stomach and thus, different mast cell populations as well.

Nitric oxide and mucosal blood flow

Endogenous NO is the smallest known bioactive product of mammalian cells, with a profound range of regulatory functions (Förstermann *et al.*, 1995, Wallace and Miller, 2000). NO acts in concert with endogenous prostaglandins and sensory neuropeptides in modulating gastric mucosal integrity (Whittle *et al.*, 1990, Wallace, 1996), including mucus secretion, blood flow, epithelial permeability and the inflammatory response (Wallace and Miller, 2000).

NO release

In 1980 a potent vascular smooth muscle-relaxing substance was discovered by Furchgott and Zawadski in the vascular endothelium and named “Endothelium derived relaxing factor” (EDRF) (Furchgott and Zawadski, 1980). It was subsequently found to be identical with NO (Palmer *et al.*, 1987, Ignarro *et al.*, 1987). Nitric oxide is produced from the amino acid L–arginine by the action of the enzyme nitric oxide synthase (NOS) and has a half–life of about six seconds (Guth, 1992). NOS can practically be found in all mammalian cells including mucosal epithelial cells, vascular endothelium, inflammatory cells and neurons of the central and enteric system. Basically, there are three isoforms of NOS in the gastrointestinal tract; namely

eNOS, nNOS and iNOS (Mashimo and Goyal, 1999, Förstermann *et al.,* 1995). Generally, eNOS and nNOS are considered to be constitutively expressed, while iNOS is an inducible enzyme requiring protein synthesis. eNOS is primarily found in the endothelium of the vasculature, nNOS in neurons (Bredt *et al.,* 1990), and iNOS in the endothelium, epithelium and inflammatory cells. iNOS is normally expressed in prolonged inflammatory conditions and recent findings suggest that it may be upregulated fairly quickly in the mucosal epithelial cells upon endotoxin (Tepperman *et al.,* 1993, Brown *et al.,* 1994, Unno *et al.,* 1997) and HPE challenge (Lamarque *et al.,* 1998). NOS-containing neurons are abundant close to submucosal arteries and in the peripheral muscle layers, but are almost absent in the mucosa (Ekblad *et al.,* 1994). Recently, an alternative source of gastric NO was proposed; a non–enzymatic NO production formed by the acidification of salivary nitrite in the stomach (Benjamin *et al.,* 1994, Weitzberg and Lundberg, 1998). This NO is aimed at protection against gastrointestinal infection by pathogenic microorganisms, e.g. *Helicobacter pylori*.

NO pathophysiology

Numerous studies have investigated the role of NO in mucosal damage (reviewed in Alican and Kubes, 1996). NOS can be inhibited by using L–arginine analogues such as N^{ω}–nitro–L–arginine (L–NNA) and nitro–L–arginine methyl ester (L–NAME), that competitively inhibit the enzyme throughout the vascular tree. Inhibition of NOS augments gastrointestinal mucosal damage by noxious agents, such as ethanol, and in acute inflammatory models on infusion of PAF or bacterial endotoxin (Hutcheson *et al.,* 1990, Alican and Kubes, 1996, Qiu *et al.,* 1996). In keeping with this NO donors improve or protect the mucosa from injury (Boughton–Smith *et al.,* 1990, Qiu *et al.,* 1996). The protective effects of NO may include maintenance of blood flow (Pique *et al.,* 1989) or possibly inducing a hyperemic response, inhibition of leukocyte and platelet aggregation, and modulating mast cell reactivity etc (Wallace, 1996, Wallace and Miller, 2000). Today, new techniques have made it possible to produce engineered animal models with the NOS gene knocked out (Mashimo and Goyal, 1999). In these animals the specific function of individual NOS isoforms can be studied. Both eNOS and iNOS are important for protection against inflammatory injury. Indeed, animals deficient in eNOS were more prone to ischemic and inflammatory injury, while iNOS-deficient animals were more susceptible to bacterial pathogens. In support of this, an increased expression of iNOS in epithelium, endothelium and lamina propria inflammatory cells was found in the gastric mucosa of *H. pylori*-positive gastritis patients (FU *et al.,* 1999). Animals deficient in nNOS had gastric vascular dilation and stasis. However, NO, in itself, could be detrimental in high concentrations (Tepperman *et al.,* 1993, Tripp and Tepperman, 1996, Unno *et al.,* 1997). It is unstable in the presence of oxygen and decomposes to yield a variety of reactive nitrogen oxide species such as peroxynitrite, nitroxyl, and nitrogen dioxide (Grisham *et al.,* 2000).

In studies of the mesentery NOS-inhibition has resulted in an increase in vascular permeability and an increase in leukocyte adhesion (Kubes and Granger, 1992, Harris, 1997). The microvascular effects elicited by the NOS inhibition may be mediated via mast cells (Kubes *et al.,* 1993) and the responsible mediators are suggested to be PAF (Arndt *et al.,* 1993, Kurose *et al.,* 1993, Kanwar *et al.,* 1994) and histamine (Kanwar *et al.,* 1994). The effects could be attenuated by pretreatment with mast cell stabilizers such as ketotifen and the PAF receptor-antagonist WEB2086. Recent reports suggest, however, that the vascular effects observed upon NOS inhibition may be the result of the surgical intervention itself (László *et al.,* 1999, László and Whittle, 1999, Pávó *et al.,* 2000). Indeed, endogenously released NO, presumably from eNOS, may be a necessary physiological defense against the deleterious effects of the inflammatory mediators released upon abdominal surgery and intestinal manipulation. Hence, inhibiting

NOS and NO production would only serve to unmask these vascular effects. Fändriks *et al.* (1997), found that luminal exposure to *H. pylori* water extracts increased the concentrations of asymmetric dimethyl arginine (ADMA), an L–arginine analog, in the extract and in the duodenal mucosa. ADMA inhibits NOS and hence, *H. pylori* may have the ability to interfere with the modulation of mucosal functions, including microvascular integrity and cytotoxicity, by reducing local NO production.

Helicobacter pylori

Helicobacter pylori is a unique gram negative, spiral shaped bacterium that thrives in the harsh hostile acidic environment in the stomach (Kelly, 1998). Today, *H. pylori* is associated with active chronic gastritis, gastroduodenal ulcers and gastric cancer (DeCross and Marshall, 1993, Genta, 1995, Genta, 1997). The pathophysiology of these disease states has not been fully understood and the early events of colonization are still obscure. Lately, questions have been raised as to if *H. pylori* is a pathogen or a commensal with possible host-beneficial effects (Labenz *et al.,* 1997, Chow *et al.,* 1998, Pütsep *et al.,* 1999). Thus, "*thumbs up*" or "*thumbs down*" to complete eradication of the bacteria is a current issue for debate (Blaser, 1997a), as is the clinical management of the infection (Falk, 1996, Moss *et al.,* 1998). An important aspect in this debate is the heterogeneity of *H. pylori*, which infers that an individual may be simultaneously infected with several strains of the bacteria with diverse properties (Blaser, 1997, Enroth *et al.,* 1999).

Short history

The history of the association between *H. pylori* and man probably goes back over a hundred thousand years. But the story of the spiral shaped bacterium and gastric ulcers started 1875 with the discovery of bacteria in ulcer margins by Bottcher and Lettule (reviewed in Kidd and Modlin, 1998). Over the following decades several studies described bacteria associated with gastric disease, and bacteria-induced ulcers were studied in experimental animals. However, no conclusive results were obtained until 1982, when John Warren and Barry Marshall discovered what they called *Campylobacter pyloridis* (Warren and Marshall, 1983, Marshall and Warren, 1984). The following year they tried to fulfill Koch's third and fourth postulates for the bacteria, i.e., that isolated bacteria could infect and colonize a histologically normal mucosa and induce gastritis (Marshall *et al.,* 1985). Marshall, a healthy volunteer, ingested a bacterial "cocktail" and could histologically confirm the association between *C. pyloridis* and the development of acute gastritis. Another group repeated the same study with similar results (Morris and Nicholson, 1987). In the late 1980's, the name of the bacteria was subsequently changed to *Helicobacter pylori*, when its taxonomic features suggested that it did not belong to the *Campylobacter* genus (Goodwin *et al.,* 1989).

Epidemiology

H. pylori is a wide-spread international bacterium that, today, is harbored in approximately 50% of the worlds population, with the highest prevalence in the developing countries. About 10% of *H. pylori* positive individuals develop serious gastric disease (gastroduodenal ulcers and cancer), while the others are asymptomatic carriers. The bacterium is commonly acquired during childhood and follows the individual as a chronic infection throughout life. A connection has been found to low socioeconomic status, although other environmental and genetic factors certainly predetermine the risk for acquisition and the individuals susceptibility to infection with the bacteria (Graham *et al.,* 1992, Enroth, 1999). However, how the bacteria are transmitted between host has not been fully clarified although the oral–oral and fecal–oral routes may be the major pathways. An effective treatment and eradication has been

achieved employing an acid-suppressive drug (omeprazole or lansoprazole) in combination with two antibiotics (clarithromycin or amoxicillin and metronidazole), the so-called “triple therapy”. Following eradication of the bacteria the probability for re-infection and relapse is very low (Enroth, 1999).

H. pylori *pathogenicity*

The bacteria normally resides in the mucus gel layer close to the epithelial surface and approximately 20% adhere to the mucosa in a process associated with cytoskeletal dearrangements and “adherence pedestal formation” (Newell, 1991, Wadström *et al.,* 1996). The bacteria are numerous in the gastric pit regions of the gastric mucosa and are also found in the intercellular crevices between the epithelial cells (Newell, 1991). The human blood group antigen Lewis^{b} is an epithelial cell surface-expressed receptor that *H. pylori* binds to in the upper third of the gastric crypt units (Borén *et al.,* 1993, Borén *et al.,* 1994, Falk, 1996, Wadström *et al.,* 1996). Although not generally accepted, it has been demonstrated that part of the bacteria’s immune and treatment-elusive properties can be attributed to its internalization into the epithelial cells (Löfman *et al.,* 1997, Engstrand *et al.,* 1997, Su *et al.,* 1999).

A large number of general and specific virulence factors have been implicated in the pathogenicity of *H. pylori* (Newell, 1991, DeCross and Marshall, 1993, Figura, 1997, Figura, 1997a, Schraw *et al.,* 1999). These include motility, the urease enzyme, mucolytic enzymes, lipopolysaccharide (LPS), adhesins, cytotoxin and immunologic escape. Motility is gained by the presence of one to six sheathed polar flagella, which are a prerequisite for successful colonization (Kelly, 1998). One of the most important and best-characterized virulence factors is the 95kDa vacuolating cytotoxin, VacA (Telford *et al.,* 1994, Phadnis *et al.,* 1994, Cover, 1996). VacA causes the formation of acidic vacuoles in epithelial cells (Cover *et al.,* 1990, Catrenich and Chestnut, 1992, Figura, 1997, Reyrat *et al.,* 1999). It may also loosen tight junctions (Newell, 1991, Reyrat *et al.,* 1999) and form anion channels in the cell membrane (Reyrat *et al.,* 1999). A putative role would be to provide a nutrient rich environment for the bacteria. VacA is cleaved *in vitro* into a 37kDa and 58kDa fragment (Figura, 1997a, Reyrat *et al.,* 1999). The latter fragment is responsible for adherence, while the former mediates the biological activity. Another protein is the cytotoxin-associated gene protein, CagA, coded for by a gene in the cag pathogenicity island (cag PAI) of the bacteria, which contains several genes homologous to virulence genes of classical pathogenic bacteria (Figura, 1997a). Only recently it has been shown that the immunodominant protein, CagA, is delivered into the epithelial cells and is likely to play a major role in *H. pylori*–host cell interactions and pathogenesis (Segal *et al.,* 1999, Stein *et al.,* 2000). The other genes are thought to induce secretion of the cytokine interleukin–8 (IL–8), from the epithelial cells, with vast inflammatory effects (Crabtree, 1996, Figura 1997a). Xiang *et al.* (1995), have proposed a classification of the bacterial isolates into two broad types, type I (56% of isolates) and type II (16% of isolates). Type I bacteria express biologically active proteins of both VacA and CagA and are associated with a higher inflammatory and ulcerogenic potential. Type II bacteria completely lack the cag PAI and produce an inactive VacA protein. Intermediate types have a dissociated expression of VacA and CagA and may have a partly deleted cag PAI.

The urease produced by *H. pylori* hydrolyses urea to ammonia. The ammonia may be important in maintaining a near neutral environment around the bacteria (Weeks *et al.,* 2000) and may also have toxic effects on the epithelial cells (Smoot and Resau, 1990). The *H. pylori* endotoxin LPS is approximately 2000 times less potent than that from *E. coli* (Perez–Perez *et al.,* 1995) but yet, possesses unique biological properties. The outer O–specific chain of the LPS mimics the Lewis^{b} blood group antigen (“antigenic mimicry”) and contributes to

camouflaging the bacteria and eluding the immune system (Moran, 1996, Appelmelk *et al.,* 1997). *H. pylori* LPS furthermore disturbs the interaction between the epithelial cells and the mucus components, thereby destabilizing the mucus barrier (Piotrowski, 1998). A surface soluble protein other than LPS has been shown to activate monocytes (Mai *et al.,* 1991). Indeed, apart from the factors mentioned above *H. pylori* secretes a heat and acid stable factor, of less than 3000 in molecular weight, that has a chemotactic activity for neutrophils and monocytes (Craig *et al.,* 1992). A recent report suggests that *H. pylori* secretes a peptide with anti–bacterial qualities, which could function as a protection against other gastrointestinal pathogens in asymptomatic *H. pylori* carriers (Pütsep *et al.,* 1999).

Animal models of H. pylori *infection*

Several animal models (Engstrand, 1995), including rats (Li *et al.,* 1998, Li *et al.,* 1999) and mice (Konturek *et al.,* 1999) have been used to try to mimic the natural course of an infection with *H. pylori* in man. In the aforementioned rat model, a chronic infection could be established with mild to moderate mucosal inflammation (Li *et al.,* 1999). This has allowed studies of ulcer healing (Li *et al.,* 1998) and in this thesis, gastric mucus gel accumulation *in vivo*. With the dawning of new technology, the interactions between *H. pylori* and the host can now be studied in genetically modified mice. An exciting new mouse model expresses the human lewis^{b} antigen on the gastric surface epithelial cells, thereby enhancing *H. pylori* adherence and infection (Falk *et al.,* 1995). This opens the door for studies of more specific aspects of the molecular pathogenesis of diseases caused by *H. pylori* infection.

MATERIALS AND METHODS

Animals and anesthesia

Male Lewis/DA F1 hybrid rats weighing 180–250g, male Wistar rats 200–230g and female Sprague Dawley rats 200–260g were used. The animals were housed under standardized conditions of temperature and illumination (12-h darkness/light periods with normal day rhytmicity) with free access to standard pelleted food and tap water. Approximately 18 hours prior to the experiments the animals were placed in cages with mesh bottom and deprived of food but had access to fresh tap water ad libitum. All animals were anesthetized by an intraperitoneal injection of 120 mg kg^{-1} bw of the barbiturate INACTIN® (Na-5-ethyl-1-(1'-methyl-propyl)-2-thiobarbituric acid). An extra dose of 10–20% of the barbiturate was given if required to ensure proper anesthetic depth. To minimize stress the animals were always fasted in groups of two and the animal house personnel familiar to the animals administered the anesthetic drug. In addition, all purchased animals were allowed at least 1 week to adjust to the new environment. By minimizing the pre–experimental stress its influence on different physiological parameters and in particular stress-related changes in the gastric mucosa were kept at a minimum. Inactin has been shown to give a stable long-term surgical anesthesia, however, with a suppressed control of body temperature and possible cardiovascular effects, such as decreased cardiac output and reduced tissue blood flow (Buelke–Sam *et al.*, 1978, Walker *et al.*, 1983, Flecknell, 1996). A rectal thermistor probe connected to a heating pad and temperature regulating unit was used to maintain body temperature at 37–38°C. All procedures performed on the animals were previously approved by the Swedish Laboratory Animal Ethical Committee in Uppsala, and were conducted in accordance with the guidelines of the Swedish National Board for Laboratory Animals.

Surgical procedure

The rats were tracheotomized with a cannula inserted into the trachea, just below the thyroid gland, to facilitate spontaneous breathing. The right femoral artery was cannulated with a polyethylene cannula containing heparin (12.5 international units (IU) ml^{-1}) dissolved in isotonic 0.9% saline (155mM NaCl), and connected to a strain gauge pressure transducer for continuous blood pressure measurements. A cannula was also placed in the right femoral vein for infusion of a modified Ringer's solution or the drugs used in the experiments. The modified Ringer's solution, contained 120mM NaCl, 2.5mM KCl, 25mM $NaHCO_3$ and 0.75mM $CaCl_2$, and was given at a rate of 1ml per h, to prevent dehydration and to maintain a normal acid–base balance in the animals.

Tissue preparation

Corpus

The stomach was exteriorized through a midline abdominal incision after cutting the gastro–hepatic ligaments and the short gastric artery from the spleen (Holm–Rutili and Öbrink, 1985). The stomach was opened by a midline incision through the forestomach with an electric microcautery instrument and any luminal contents gently flushed out with warm 0.9% saline. The rat was placed on its left side on a heating pad on a Lucite microscope stage and the stomach was gently everted and draped luminal side up, over a truncated cone in the middle of the table (Fig. 2). To prevent the stomach from slipping off it was held in place by two pins inserted through the forestomach and fastened in rubber rings at the base of the cone. A

mucosal chamber with a hole (diameter 1.2 cm^2) in the bottom, corresponding to the position of the cone, was carefully placed over the exposed mucosa.

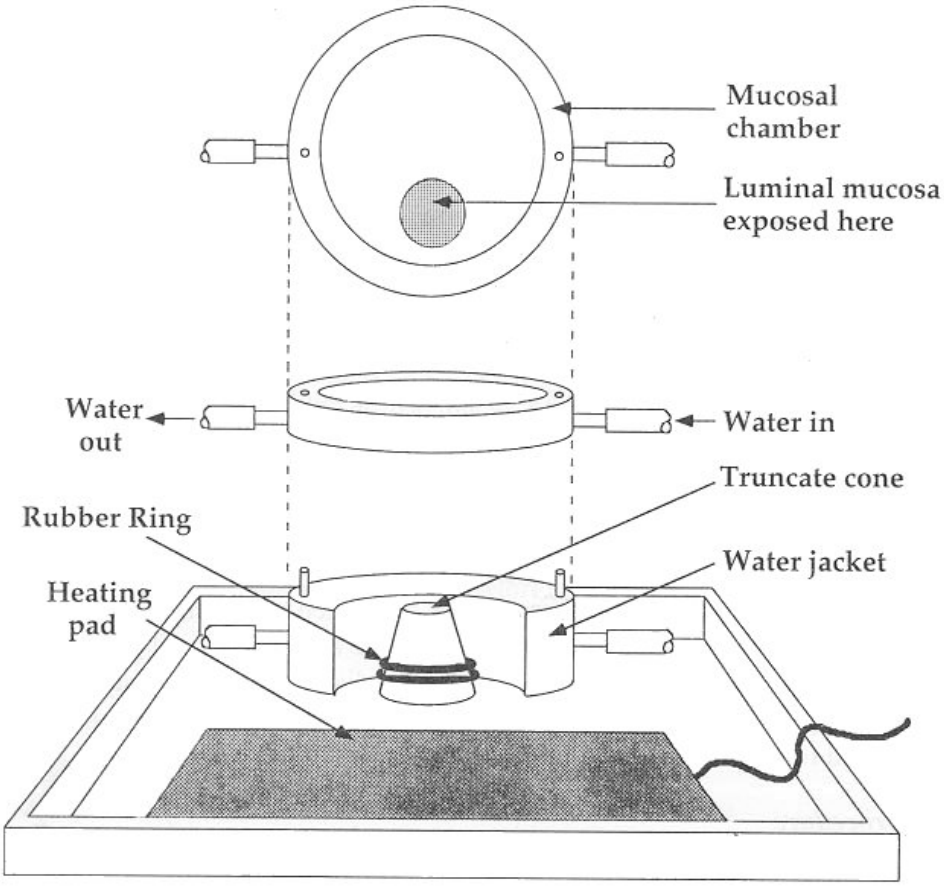

Figure 2. *Schematic drawing of the Lucite microscope stage used to mount the stomach for intra–vital microscopy. A modified stage with three small pins at the back top edge of the truncated cone was used for the intestine.*

Antrum

To study the antrum the above procedure was employed with a slight modification. Following exteriorization, the stomach was opened with a 1–2 cm incision through its ventral side, close to and along the greater curvature from the corpus–antrum transition zone and approximately half–way up through the corpus. Care was taken to avoid damaging the Rt. gastroepiploic arteries along the greater curvature, and the use of an electric microcautery instrument minimized bleeding from smaller blood vessels in the corporal mucosa. The antrum was mounted over a smaller truncated cone than that for the corpus and a mucosal chamber (hole diameter 0.9 cm^2) gently placed over the exposed mucosa. The tissue was held in situ by a pin inserted through corpus and fastened in the rubber rings at the base of the cone.

Intestine

All studied parts of the intestine were opened with a 2–3 cm midline incision through the anti–mesenteric border using an electric microcautery instrument. The ventral side of the intestine was draped luminal side up onto the truncated cone of a Lucite microscope stage and a mucosal chamber (hole diameter 0.9 cm^2) gently placed over the tissue. The mounted tissue

was held in situ by fastening the incision edge on three small pins at the top edge of the cone. The duodenum was opened approximately two centimeters distal to pylorus. Pancreaticobilary secretions were prevented from entering the preparation by ligating and cannulating the common bile duct close (2–3mm) to its entrance into the duodenum. The jejunum was opened 10 cm distal to pylorus, ileum 5 cm proximal to caecum and colon 5 cm distal to caecum.

During tissue preparation care was taken to avoid twisting or stretching the stomach or intestine, as this markedly impaired blood flow and the general condition of the tissue. There remained an approximately 2-mm gap at the junction between the mucosal chamber and the tissue, which was sealed with silicone grease to avoid detrimental pressure on the tissue. All tissues were kept warm and moist during the preparation procedure by bathing them with warm saline. The double-bottomed mucosal chamber was filled with 5–7 ml of warm 0.9% unbuffered saline, acid (Paper II) or the water extract from *H. pylori* (Papers II, III and IV), and the solution was kept warm (37°C) by means of warm water perfusing the bottom of the chamber. The mounted tissues were placed under a Leitz stereomicroscope and the exposed areas were transilluminated with a 150-W optic fiber-guided light source.

Bacterial strains and growth conditions

H. pylori strain 88–23, wildtype was kindly provided by M. Blaser Nashville, Tenn., USA and strain A5, wildtype, and its isogenic mutant A5VacA (VacA$^-$ and CagA$^-$) were both kindly provided by L. Janzon, AstraZeneca, Södertälje, Sweden. These strains were both of type I (Xiang *et al.*, 1995). The bacteria were grown on Gonococcal Chocolatised (GC) agar plates and incubated at 37°C in a triple gas incubator under moist microaerophilic conditions (85% N_2, 10% CO_2 and 5% O_2).

H. pylori strain Hel73 used for inoculation of the animals in paper II was also a wildtype strain. The isolation procedure and mouse adaptation of the bacteria, and the characteristics of the rat model with the established *H. pylori* infection, have been described in detail (Li *et al.*, 1998, Li *et al.*, 1999). In brief, the bacteria were grown in brucella broth (pH7.0) supplemented with 10% fetal calf serum for 24 h at 37°C under microaerophilic conditions as described above. The *H. pylori* suspension ($5x10^6$–$5x10^8$ CFU ml^{-1}) was given to the rats by gavage (2 ml per rat) twice daily, with an interval of 4 h, for two consecutive days. Three hours before the first inoculation and once daily during the following 6 days, the rats were given omeprazole (400µmol kg^{-1} bw by gavage) suspended in carbonate buffered 0.5% Hydroxypropylmethylcellulose®, pH9.

The wild type *E. coli* strain, ATCC–25922, was cultured at 37°C in a Mueller Hinton broth containing $CaCl_2$ (50 mg l^{-1}) and $MgCl_2.6H_2O$ (50 mg l^{-1}). Growth of *H. pylori* and *E. coli* was checked by Gram stain before use.

Preparation of the bacterial water extracts

The procedure for the preparation of the water extract from *H. pylori* (HPE) is a modification of that by Xiang *et al.* (1995), and is described in detail in paper III. In brief, three day cultures of *H. pylori* were harvested and suspended in sterile distilled water (about 10^9 colony-forming units (CFU) ml^{-1}) for 30 min at room temperature. Water-soluble components were separated from cell remnants by ultracentrifugation and filtration through 0.2-µm syringe filters. The broth containing *E. coli* was harvested after 6.5 h, at which time it contained approximately 5

x 10^8 CFU ml^{-1}. The broth was centrifuged and the pellet used to prepare a water extract (ECE) as described above.

Application of the bacterial water extracts (Papers II–IV)

The concentrated water extracts were diluted twice with a 1.8-% saline solution to obtain an isotonic solution. The pH of the solution, normally 7.8–8.2 for HPE and 6.1–7.2 for ECE, was then adjusted to the pH of the 0.9% saline solution (approximately pH 6.0) used in the experiment. In paper III the HPE was boiled at 100°C for 30 min to inactivate any heat sensitive factors in the extract. The extracts were applied for 40 min in all groups followed by a 40-min period with 0.9% saline.

Normally, colonizing *H. pylori* are attached to the mucosal epithelium and there release their virulence factors, in addition to having direct effects on the epithelial cells. The juxtamucosal release of the virulence factors confers an immediate access to the mucosa. In the in situ model employed in papers II–IV, the mucus layer was therefore removed before application of the warm (37°C) extracts in an attempt to mimic the *in vivo* situation.

Removal of the gastrointestinal mucus gel layer

In all papers the gastrointestinal mucus gel layer was removed before application of bacterial water extracts (Papers II–IV), acid (Paper II) and/or measurement of mucus gel renewal rates (Papers I and II). The mucus gel layer was translucent and the surface was visualized by applying a small amount of carbon particles suspended in 0.9% saline to the mucosal chamber. The mucus layer was then carefully sucked off using a small polyethylene cannula connected to a weak vacuum suction pump during observation through a stereomicroscope. During this procedure contact with the epithelium was avoided. These attempts were only in part successful as a firmly adherent mucus layer (described in paper I) remained attached to the mucosa and was impossible to remove by further suction or mechanically in pilot experiments with moist cotton swabs.

Mucus gel thickness measurements (Papers I and II)

Mucus gel thickness was measured using a micropipette held by and maneuvered with a micromanipulator (Fig. 3). The micropipettes were manufactured by pulling borosilicate glass tubing (outer diameter 1.2 mm and inner diameter 0.6 mm) in a pipette puller to a tip diameter of 1–2 µm. The tips of the pipettes were dipped into a solution containing 75% silicone and 25% acetone, and dried at 100°C for 30 min to ensure a non–sticky surface. In this way repeated measurements could be made without the mucus gel adhering to the pipette and subsequently tearing the mucus gel layer. To visualize the otherwise translucent mucus gel surface, carbon particles were instilled onto the gel. Using the micromanipulator, the tip of the micropipette was placed on the surface of the gel and pushed through the gel layer at an angle (a) of 25–45° to the mucosal surface. The distance (l) from the mucus gel surface to the mucosal surface was measured with a ”digimatic indicator” attached to the micromanipulator. Measurements were made at 3 to 7 different sites or villus tips, and each position was registered and used throughout the experiment. The mean value of all measurements on every measurement occasion was taken as one thickness value. The actual mucus gel thickness (T) was calculated using the formula: $T = l \times \sin a$. Mucus renewal rates were determined by measuring total mucus thickness at regular intervals (15 min in the intestine and 20 min in the stomach). Measurements were made over a 90- (intestine) or 80-min (stomach) period, before (Paper I) and after removal of as much of the adherent mucus gel layer as possible (Papers I and

II). The accuracy of this technique is based on the assumption that the mucosal or villus tip surface is resting in a horizontal plane at the measurement position.

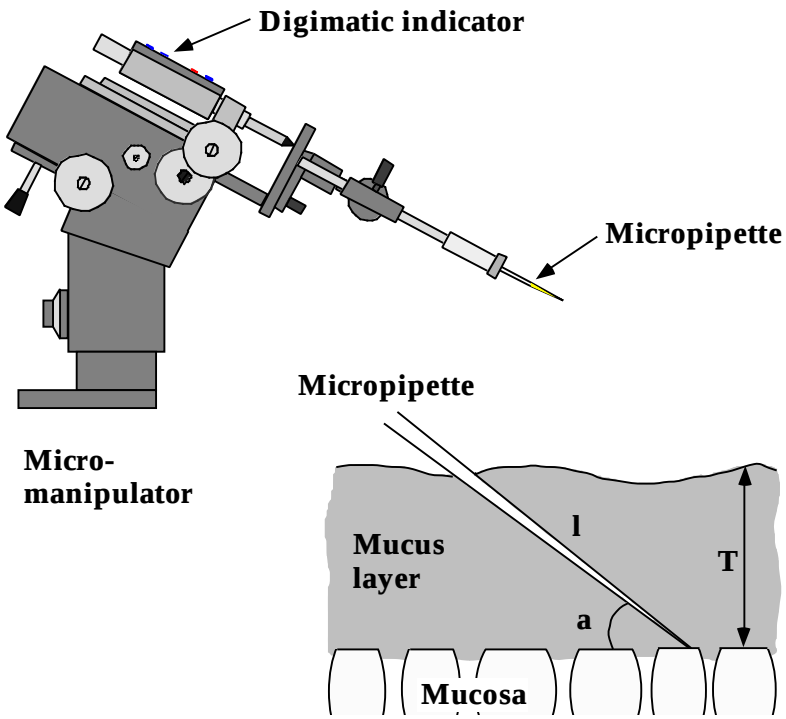

Figure 3. *Mucus thickness measurement setup.*

Laser–Doppler flowmetry (LDF) measurements (Papers III and IV)

Changes in local gastric blood flow were measured using the LDF technique (Perimed, Periflux® instruments Pf2, Pf3 and Pf4001) (Fig. 4). In 1975, Stern was the first to report the possible use of the laser–Doppler technique for circulatory studies. Red monochromatic laser light (wavelength 633 nm) is guided to the tissue by a glass optic fiber (diameter 0.7 mm). In the tissue the light beam is scattered on moving cells, such as red blood cells, and on static structures (Fig. 4). The light beams scattered on moving objects undergo a frequency shift/broadening according to the Doppler effect, while the beams scattered by static structures alone remain unshifted in frequency (Nilsson *et al.*, 1980a). The magnitude of the Doppler-shift is dependent on the angle of reflection of the incident beam, velocity and propagation direction of the moving object, and the number of successive Doppler shifts (Nilsson *et al.*, 1980a, Tenland, 1982). A portion of the back-scattered light is guided back by a number of equal-sized glass optic fibers (diameter 0.7 mm) to a photo detector and signal processor. The mixed signal is processed into an output voltage signal with a magnitude dependent on the number and the velocity of moving blood cells, blood cell flux, in the illuminated tissue volume (Nilsson *et al.*, 1980a, Nilsson 1984). Blood cell flux can be linearly correlated to the output signal based on two facts: the portion of back-scattered light that has undergone Doppler shift is approximately linearly related to the volume fraction of moving red blood cells; and the mean Doppler frequency is linearly correlated to the average red cell

absolute velocity (Tenland, 1982). This linear relationship is true for both high- and low flow rates and blood-cell volume-fraction (Nilsson, 1984). The LDF technique permits continuous and repeated linear recordings of red blood cell flux. This has been strongly correlated to tissue blood flow in several studies, in the gastrointestinal tract and other tissues, when compared with other measurement techniques (Nilsson 1980b, Ahn *et al.*, 1985, Kvietys *et al.*, 1985, Granger and Kvietys 1985, Smits *et al.*, 1986, Holm–Rutili and Berglindh 1986, Ahn *et al.*, 1988, Allen *et al.*, 1988).

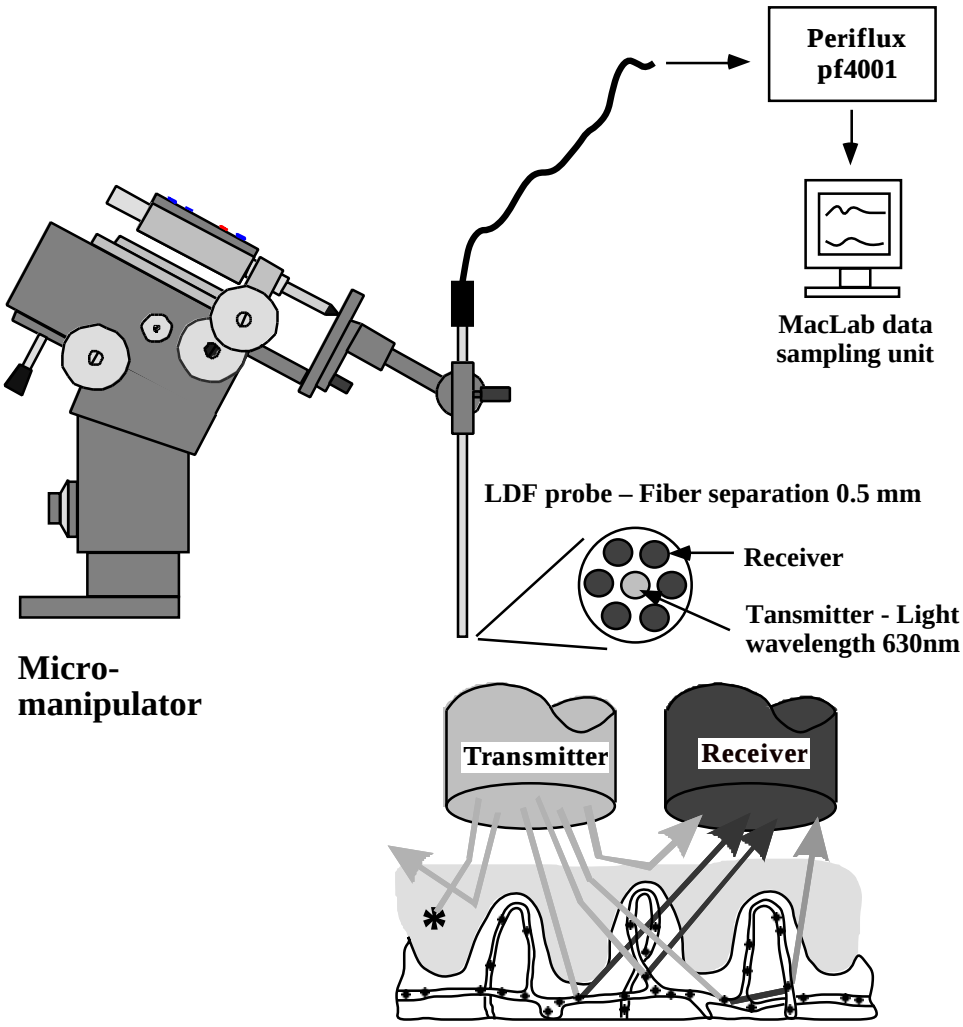

Figure 4. *Laser–Doppler flowmetry (LDF) setup and the principles behind blood flow measurement with the LDF technique. Possible pathways for the light rays in the skin are illustrated at the bottom. These include absorbed (*), Doppler-shifted and non Doppler-shifted rays.*

The glass optic fibers are gathered in a probe (fiber separation 0.5 mm) held by a micromanipulator in a fixed position approximately 1 mm above the luminal mucosal surface (Fig. 4). The probe was submerged in the solution bathing the mucosa thereby minimizing surface reflection of the laser light.

At what depth is blood flow measured? The measuring depth of the probe has been a matter of debate. The penetration depth is dependent on the wavelength of the laser light, separation distance between the fibers in the probe and the tissue properties. A probe similar to that used in these studies, was previously observed to have a penetration depth of at least 6 mm in feline and human gastrointestinal tissue (Johansson *et al.*, 1987). Thus, in the present studies, blood flow most probably was measured through the entire wall of the illuminated portion of the rat stomach (approximately 2 mm thick). However, the registered blood flow is mainly mucosal, since the amount of back–scattered light decreases exponentially with the depth in the tissue and about 80% of the blood flow to the stomach perfuse the mucosa.

Blood flow in these studies is expressed in relative terms as a percentage of baseline values (% of control) in 5–10 min periods. Before use, the probes were calibrated in a standardized calibration solution (Perimed) equivalent to 250 perfusion units (PFU), irrespective of the magnitude of the actual voltage signal. By combining different techniques and using calibrated probes, LDF output signals expressed as voltage or PFU can be approximately interpreted as absolute blood flow units (Ahn *et al.*, 1985) and may be compared between tissues and experiments.

A potential drawback with this technique is that lateral tissue- or probe movement changes the illuminated volume and may influence the recording. In the rat stomach blood flow was however found to be fairly similar over the exposed area and minor probe movements did not notably affect the recording.

Acid secretion (Papers II–IV)

Acid secretion was measured by backtitrating the solution bathing the mucosa at regular intervals of 10–20 min. The solution retrieved from the chamber was titrated with 1 or 10 mM NaOH to the initial pH of the applied 0.9% saline solution. Acid secretion is presented as micro–equivalents of hydrogen ions secreted per min and cm^2 of the exposed (1.2 cm^2) mucosa ($\mu Eq\ min^{-1}\ cm^{-2}$).

Administered drugs (Paper IV)

L–NNA is a nitric oxide synthase (NOS) inhibitor. It is an L–arginine analog that competes for the NOS enzyme and provides an unspecific inhibition of endogenous NO production. L–NNA was administered as an intravenous bolus dose (10 mg kg^{-1} bw in Ringer's solution) followed by a continuous infusion (3 mg kg^{-1} bw in Ringer's solution). The dose markedly increased mean arterial blood pressure (MAP) by approximately 30 mm Hg, and tissue blood flow and acid secretion increased transiently. After 50–60 min stable values were attained. Ketotifen is generally used as a mast cell-stabilizer with inhibitory effects also on basophils and neutrophils (Craps and Ney, 1984). It was used at a dose of 100 µg 100 g^{-1} bw intravenously to stabilize mucosal mast cells. WEB2086, a PAF-receptor antagonist, was given as a single bolus dose of 5 mg kg^{-1} bw intravenously to counteract the possible microvascular effects of PAF released in the mucosa. The plasma half–life for WEB2086 is approximately 6 h in the male rat (Bar–Natan *et al.*, 1995). Neither ketotifen nor WEB2086 had any conceivable effects on registered parameters.

Statistics and calculations

The results in all papers are generally presented as mean values ± standard errors of the mean (SE). The statistical differences between data were evaluated using an Analysis of variance (ANOVA) for repeated measurements when comparing values within a group (Papers I and III)

and a factorial analysis when comparing values between groups (Papers I and II). The ANOVA was followed by the Fischer protected least-significant difference (PLSD) test. Student's T–test for unpaired measurements was used to compare single values (Papers I and II). All statistical calculations were performed on a Macintosh computer using the Statview–SE and Graphics software. P values <0.05 were considered significant.

Vascular resistance (Paper IV) was calculated by dividing the mean MAP with the mean actual LDF output value for a given time period according to Ohm's law (R=U/I equivalent to R=MAP/LDF). The values are presented as per cent of the basal control values (% of control).

RESULTS AND COMMENTS

The adherent gastrointestinal mucus gel layer: Thickness and physical state in vivo (Paper I)

In earlier studies the mucus layer in different parts of the gastrointestinal tract has been studied mainly using different in–vitro techniques. These studies have usually covered a limited part of the gastrointestinal tract and have yielded different incomparable results. Therefore, the mucus gel layer was studied with respect to thickness, physical state and rate of renewal throughout the gastrointestinal tract *in vivo*. As the same model was used, the results are comparable between the different regions of the gastrointestinal tract. Mucus thickness was measured before and after mucus removal by applied suction, which can be considered to mimic the mild shear forces during the normal digestive process.

Observation of the mucus layer through the stereomicroscope revealed that *in vivo* there is a continuous, translucent layer of adherent mucus gel in all parts of the gastrointestinal tract, from the stomach to the colon. The mucus layer had an even surface and did not follow the contours of the villi in the intestine. In a few animals a loose sloppy mucus layer covered the adherent mucus gel in the stomach and in the intestine. In general, this sloppy mucus was removed when changing solutions in the chamber or was detached by the micropipette at the first measurement occasion.

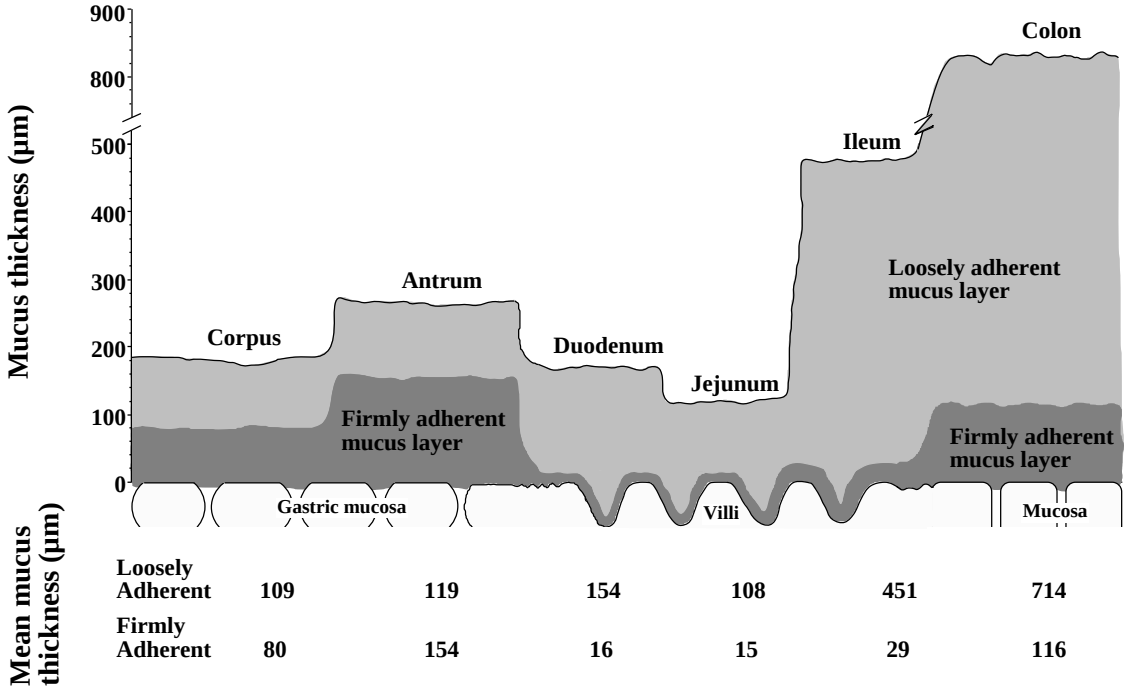

Figure 5. *Mucus thickness measured throughout the gastrointestinal tract. Values are presented as mean values.*

The thickest adherent mucus gel layers were found in the distal parts of the intestine; in the proximal colon and ileum, the mean thicknesses were 830 µm and 480 µm respectively.

Intermediate values were found in the stomach corpus and antrum, 189 µm and 273 µm respectively, while the thinnest mucus layers were found in the duodenum and jejunum, 170 µm and 123 µm respectively (Fig. 5).

About 58% in the corpus, 44% in the antrum and 86% in the colon of the adherent mucus gel layer could be removed by applied suction. The remaining firmly adherent mucus gel layer was impossible to remove by further suction or by using moist cotton swabs (in pilot experiments). The firmly adherent, shear resistant, mucus gel was a thick continuous layer over the corporal, antral and colonic mucosa with mean thickness values between 80–154 µm (Fig. 5). In contrast, in the small intestine approximately 90% of the adherent mucus layer was removed to leave a thin and discontinuous firmly adherent mucus layer with a mean thickness of approximately 20 µm, but with several tops of the villi apparently free of mucus. Interestingly, the thickness of the firmly adherent layer did not mirror the thickness of the total mucus layer in the different parts of the gastrointestinal tract.

Table I. *Mucus renewal rates in the gastrointestinal tract before (resting conditions) and after mucus removal by applied suction.*

	Before removal (µm min^{-1})	After removal (µm min^{-1})	Time Interval (min)
Corpus	≈0	0.9	80 / first 20
Antrum	≈0	1.25	80 / first 20
Mid-Duodenum	2.3	1.3	90 / 90
Proximal Jejunum	3.0	2.1	90 / 90
Distal Ileum	4.7	1.7	45 / 90
Proximal Colon	5.0	6.1	90 / 90

Values are presented as mean increase in mucus thickness per minute during the specified interval (before/after mucus removal). In the stomach the values for the period after mucus removal are for the first 20-min interval only. The increase was fairly constant in the intestine but decreased with time in the stomach.

In the stomach there was practically no increase in the thickness of the adherent mucus gel layer during resting conditions. In the intestine however, there was a continuous increase in mucus thickness at a fairly constant rate (Table I). Following mucus removal a renewal of the mucus layer ensued in all segments (Table I). The lowest renewal rates were found in the corpus and in the antrum, whereat, there also was a gradual decrease in renewal rate to almost 0 µm min^{-1} in the corpus and approximately 0.16 µm min^{-1} in the antrum after 80 min. Obversely, in the duodenum, jejunum and ileum the renewal rates were constant but slightly lower than that before mucus removal, whilst that in the colon was slightly higher (Table I). A second mucus removal revealed that the thickness of the firmly adherent gel layer was unchanged and that the observed mucus renewal was an increase in thickness of the loosely adherent gel layer alone.

Acute and chronic effects of **Helicobacter pylori** *on the gastric mucus gel* **in vivo** *(Paper II)*

There is still some controversy as to if *H. pylori* affects the mucus layer during an infection. The acute effect of HPE on the mucus gel renewal rate was investigated by luminally instilling the HPE immediately after mucus removal. In addition, the effect of a chronic *H. pylori* infection on mucus gel thickness, renewal rate and response to luminally applied acid pH1 (100mM HCl made iso–osmotic with saline) for 20 min was studied.

A luminal application of HPE from the *H. pylori* strains 88–23, A5 and A5VacA (VacA$^-$ and CagA$^-$) all caused a significantly lower renewal rate of the mucus gel in the corpus, compared to control animals, following mucus removal. A significant difference was seen in all groups 60 min after mucus removal (i.e., after 40 min with HPE and 20 min with saline). At this time, the total increase in mucus gel thickness in the HPE-treated animals compared to the controls was reduced by 51% with 88–23, 41% with A5 and 44% with A5VacA. There were no significant changes in acid secretion that could be causally linked to the HPE application.

The total mucus gel thicknesses of the corpus and antrum in the control animals, 238±39 µm and 359±73 µm, respectively, were significantly thicker than those in the animals infected with the *H. pylori* strain Hel73, 103±16 µm and 282±37 µm, respectively. Both the loosely and firmly adherent mucus layers were significantly thinner in the corpus of the infected animals. In the antrum however, only the thickness of the firmly adherent layer was significantly reduced. Basal mucus renewal rates were similar between controls and infected animals following the first mucus removal and during luminal exposure to saline. Warm acid was then applied onto the gastric antral and corporal mucosa following a second mucus removal. The ensuing mucus renewal rate was significantly increased compared to the basal rate in the antrum of the control animals, but the increase was significantly attenuated in the infected animals. Instillation of acid to the corporal mucosa did not induce any significant change in mucus renewal rate in either group. Again mucus secreted after mucus removal and in response to acid only increased the thickness of the loosely adherent layer. Acid secretion in the infected animals (≈0.01 µEq min^{-1} cm^{-2}) was not significantly lower than in controls (≈0.03 µEq min^{-1} cm^{-2}).

The results suggest that one or more *H. pylori* products, other than VacA and CagA, interfere with gastric mucus gel accumulation. It acutely reduces the rate of mucus gel renewal and subsequently total mucus thickness in the corpus, and chronically reduces the total mucus thickness in the stomach. In addition, a chronic *H. pylori* infection attenuates the rapid acid-stimulated renewal of the loosely adherent mucus gel in the antrum.

Extracts of **Helicobacter pylori** *reduce gastric mucosal blood flow through a VacA- and CagA-independent pathway in rats (Paper III)*

In this study mucosal blood flow was measured using the LDF technique to study the acute effects of HPE on the gastric microcirculation. HPE from strains 88–23, A5 and A5VacA were instilled onto the mucosa after mucus removal. With reference to the results in papers I and II, a firmly adherent layer must have remained 80–100 µm thick, which any active factor would have to penetrate to gain access to the mucosa. All HPE's significantly reduced gastric mucosal blood flow. This reduction remained even after the extract was removed and replaced with saline (Table II). The effect on the blood flow was immediate and significant after 15 min with HPE from 88–23 and after 30 min with A5 and A5VacA.

The question then arose as to if this reduction in blood flow was unique to *H. pylori*. A similar water extract from *E. coli* was prepared and instilled onto the mucosa. The result was an unaffected blood flow (Table II). This would imply that the acute reduction in blood flow was caused by a factor absent or less potent in *E. coli*. Thus, an attempt was made to inactivate all heat sensitive factors by boiling the HPE from 88–23. Applying the heat-treated extract on the mucosa had a similar effect on blood flow as that observed with the native HPE (Table II).

Table II. *Per cent reduction in gastric corporal mucosa blood flow (LDF) during and after instillation of HPE and ECE onto the mucosa.*

	Treatment		Saline	
Time (min)	20	40	60	80
Saline	0 ± 4	5 ± 4	2 ± 5	5 ± 4
88–23	12 ± 5 *	14 ± 5 *	9 ± 5	16 ± 5 *
A5	10 ± 3	17 ± 5 *	12 ± 6 *	16 ± 9 *
A5VacA	8 ± 3	13 ± 5 *	12 ± 3 *	15 ± 5 *
88–23, 100°C	15 ± 3 *	12 ± 4 *	16 ± 6 *	19 ± 5 *
E. coli	-2 ± 2	3 ± 5	7 ± 5	4 ± 7

Values are means ± SE and * P<0.05 when compared with the control level before treatment.

It was concluded that a heat stable factor in HPE other than VacA and CagA and small enough to penetrate the firmly adherent mucus layer, induces a reduction in gastric mucosal blood flow.

Helicobacter pylori ***extracts reduce gastric mucosal blood flow by a nitric oxide-independent but mast cell- and platelet-activating factor-dependent pathway in rats (Paper IV)***

In this study HPE from *H. pylori* strain 88–23 was used to further investigate the mechanisms behind the effects of HPE on the gastric mucosal blood flow. The questions addressed were if the reduction in blood flow was the direct result of a bacterial inhibition of the endogenous production of NO or if the reduction was the result of mast cell produced vasoactive factors.

The first question was answered by pre–treating the animals with L–NNA before instilling the HPE onto the corporal mucosa. A 19% reduction in blood flow was seen after 40 min and a 27% reduction seen after another 20 min with saline. Thus, the reduction is similar to that in paper III during the first 40 min with HPE, but is furthered during the following saline period.

The mast cell stabilizer ketotifen was used to evaluate the influence of mucosal mast cells. Ketotifen is predominantly used as a mast cell stabilizer, although it has been seen to have effects on other inflammatory cells. A bolus dose of ketotifen, during concomitant inhibition of NOS with L–NNA, completely inhibited the blood flow reducing effect of the HPE. Which specific factor was thus responsible for the effect? An antagonist, WEB2086, of the receptor of the potent vasoconstrictor PAF was also tested during concomitant NOS inhibition. Again, the reduction in blood flow was completely attenuated.

The results entrust that the reduction in mucosal blood flow by HPE was caused by the vasoactive properties of PAF, probably released from degranulating mast cells. The effect on blood flow was not due to an inhibition of endogenous NO, although, a NOS inhibition cannot be ruled out as a prerequisite for the actions of the HPE.

SAMMANFATTNING PÅ SVENSKA

Delarbete I

Mucus seceneras i hela mag–tarmkanalen, från magen ner till ändtarmen. Den bildar en gel, slemlagret, som täcker hela slemhinneytan och utgör en unik fysisk barriär som skyddar mot skadligt innehåll i lumen. I tidigare *in vitro* studier har forskare försökt studera detta slemlager med hänsyn till tjocklek och fysiska egenskaper under normala och patologiska förhållanden. Samtliga studier har genererat olika resultat och ofta endast avsett ett begränsat segment av mag–tarmkanalen. Syftet med detta arbete var att studera tjockleken, utseendet och tillväxttakten av slemlagret genom hela mag–tarmkanalen i en *in vivo* modell för att erhålla resultat som är jämförbara mellan mag–tarmkanalens olika delar.

Hanråttor sövdes med Inactin^{â} och magsäcken eller tarmsegementet frilades, öppnades i förmagen respektive anti–mesenteriellt och monterades med slemhinnan uppåt på ett plexiglasbord för intravitalmikroskopi. Slemlagret studerades genom ett stereomikroskop och slemlagrets tjocklek mättes med hjälp av mikropipetter som fördes in i slemlagret med hjälp av en mikromanipulator. Slemtjockleken mättes under en tidsperiod före och efter det att slemlagret försiktigt sögs bort. Resultaten visade att slemlagret var genomskinligt och heltäckande över slemhinneytan i alla delar av mag–tarmkanalen. Slemlagret var tjockast i kolon (≈830 µm) och ileum (≈480 µm) med intermediära värden i magsäcken och tunnast i jejunum (≈123 µm). När slemlagret sugits bort återstod ett lager med fast, segt slem i magsäcken (≈80 µm i korpus och 154 µm i antrum) och kolon (≈154 µm) som var heltäckande och omöjligt att suga bort. I tunntarmen däremot, så var detta kvarvarande slemlager mycket tunt (≈20 µm) och saknades helt på enstaka villi. Efter slemavsugningen startade en snabb återtillväxt av slemlagret i samtliga delar av mag–tarmkanalen. Tillväxten var långsammast i magsäcken och snabbast i kolon. I tunntarmen var slemlagrets återtillväxt lika stor i alla delar.

Sammanfattningsvis visade denna studie att slemlagret i mag–tarm kanalen är genomskinligt, heltäckande och betydligt tjockare än det som tidigare visats i *in vitro* studier. Slemlagret består av två stycken lager — ett yttre löst adhererande lager och ett inre fast adhererande lager. Det yttre lagret kan lätt tas bort genom försiktig avsugning.

Delarbete II

Magsäckens slemlager utgör en viktig barriär mot de skadliga ämnen som finns i lumen. Dess tjocklek och fysiska egenskaper är viktiga för dess funktion. ”*Magsårsbakterien*” *Helicobacter pylori* har i tidigare studier visats förändra slemlagrets struktur och påverka dess frisättning från mucusproducerande celler *in vitro*. En annan studie har i motsats till detta visat att slemlagrets viskositet ökat vid *H. pylori* infektion. Det är tydligt att det fortfarande råder oklarhet om huruvida *H. pylori* påverkar slemlagrets egenskaper vid en infektion *in vivo*. I detta arbete studerade vi de akuta effekterna av virulensfaktorer utsöndrade från *H. pylor*i och de kroniska effekterna av en etablerad *H. pylori*-infektion på slemlagrets tjocklek och tillväxt *in vivo.* Vi studerade även hur luminalt tillsatt syra påverkar slemtillväxten i kroniskt infekterade djur.

Magsäcken i Inactin^{â}-sövda råttor frilades, öppnades och korpus eller antrum monterades med lumensidan uppåt för intravitalmikroskopi. Slemlagrets tjocklek mättes före och efter avsugning med mikropipetter som med en mikromanipulator fördes ner i slemlagret. De akuta

effekterna av *H. pylori* studerades i djur som luminalt utsattes för ett vattenextrakt av *H. pylori* (HPE) från stammarna 88−23 (vildtyp), A5 (vildtyp) samt A5VacA, en mutant som saknade produktion av virulensfaktorerna VacA och CagA. Bakterier som uttrycker dessa proteiner kallas för typ I och har en förhöjd inflammatorisk potential. Till de kroniska försöken användes djur som fyra månader tidigare innoculerats med en suspension av *H. pylori* (stam Hel73). Samtliga HPE hämmade slemlagrets tillväxt i korpus jämfört med kontrollråttor. I de kroniskt infekterade djuren var slemlagrets tjocklek, 103 ± 16 µm i korpus och 282 ± 37 µm i antrum, signifikant tunnare än i kontrolldjur, 238 ± 39 µm i korpus och 359±73 µm i antrum. Även det fast adhererande slemlager som återstod efter slemavsugningen (se delarbete I) var signifikant tunnare i de infekterade djuren. Vid luminal applicering av syra pH1, var den efterföljande tillväxten av slemlagret hämmad i de infekterade djuren jämfört med kontrollerna.

Denna studie har visat att *H. pylori* hämmar tillväxten av slemlagret i korpus, *in vivo,* genom en mekanism som är oberoende av bakteriens virulensfaktorer VacA och CagA. Slemlagrets totala tjocklek och tjockleken på det fast adhererade slemlagret förtunnades vid en kroniskt etablerad infektion. Även tillväxten i respons till luminal syra är hämmad.

Delarbete III

Magsäcken utsätts ständigt för olika skadliga ämnen, däribland den egensecenerade syran och enzymet pepsin, som båda behövs för nedbrytning av proteiner i födan. Blodflödet i slemhinnan spelar en viktig roll i tillförseln av näringsämnen till magslemhinnans celler, samt för utspädning och bortforsling av toxiska slaggprodukter och andra ämnen som lyckats ta sig ner i slemhinnan. Av detta framgår, att en hämning av blodflödet väsentligen skulle försämra slemhinnans fuktion och därmed skydd mot skadliga ämnen. Bakterien *Helicobacter pylori* har visats sänka blodflödet i patienter med en kronisk infektion. Mekanismen bakom denna effekt är dock fortfarande oklar. Syftet med denna studie var att undersöka den akuta effekten av vattenlösliga virulensfaktorer från *H. pylori* på slemhinnans blodflöde samt syrasekretion. De faktorer som studeras speciellt benämns VacA och CagA (se delarbete II).

Magsäcken i Inactin^{å}-sövda hanråttor (LxDA) frilades och korpusdelen monterades med slemhinnan uppåt för intravitalmikroskopi. Blodflödet mättes med laser–Doppler flödesmetri (LDF). Denna teknik ger inga absoluta blodflödesvärden, utan erhållna värden används som ett mått på procentuell förändring i blodflödet jämfört med en kontrollnivå under basala förhållanden. HPE producerades från stammarna 88−23, A5 samt A5VacA (se delarbete II). Ett extrakt gjordes även från bakterien *Escherichia coli* (ATCC–25922). Innan extrakten sattes till slemhinnan, så avlägsnades så mycket som möjligt av slemlagret för att underlätta för bakterieprodukterna att ta sig ner till slemhinnan. Kvar fanns dock alltid ett fast adhererande slemlager närmast slemhinnan som inte gick att ta bort (se delarbete I). Alla HPE sänkte blodflödet med 15–19% medan extraktet från *E. coli* inte hade någon effekt på blodflödet. Sänkningen av blodflödet kunde inte förhindras genom att koka extraktet. Syrasekretionen höjdes något av extrakten från A5 och A5VacA men inte av det från 88–23.

Dessa resultat visar att *H. pylori* kan försämra magslemhinnans normala förmåga att skydda sig genom att också akut sänka blodflödet i slemhinnan. Den ansvariga faktorn måste vara relativt liten, då den kan ta sig igenom det fast adhererande slemlagret, och vara värmestabil. Virulensfaktorerna VacA och CagA är inte nödvändiga för sänkningen av blodflödet som verkar specifik för *H. pylori*, eftersom den ej ses med ett extrakt från *E. coli*. De olika effekterna på syrasekretionen kan spegla skillnader mellan *H. pylori*stammarnas

verkningsmekanismer. Men avsaknaden av en effekt kan också bero på att de celler (G– och D–celler) som reglerar syrasekretionen och som tidigare visats påverkas av *H. pylori*, sitter i antrum som inte nås av extraktet i denna försöksuppställning.

Delarbete IV

H. pylori har visats hämma blodflödet i magsäcken akut (se delarbete III) och kroniskt. Det har rapporterats, att en faktor som produceras av bakterien eller som produceras i slemhinnan vid infektion kan hämma den endogena produktionen av kväveoxid (NO). NO har en blodkärlsvidgande effekt och är viktig för bibehållande av ett adekvat bloodflöde i vävnaden. I slemhinnan finns också mastceller som frisätter ett flertal inflammatoriska ämnen, däribland ”*platelet activating factor*” (PAF). PAF är en potent blodkärlskonstriktor och kan också aktivera blodplättar som klumpar ihop sig och ”korkar igen” blodkärlen. Syftet med denna studie var att undersöka om den endogena NO-produktionen hämmades av *H. pylori* och om denna effekt i sig var tillräcklig för en hämning av blodflödet. Vidare undersöks om PAF frisatt från aktiverade mastceller kan vara involverad.

Hanråttor (LxDA) sövdes med Inactin och blodflödet i korpus mättes med LDF tekniken. Samtliga djur förbehandlades med kväveoxidsyntashämmaren L–NNA, som ospecifikt hämmar all produktion av NO. En grupp gavs också mastcellsstabilisatorn ketotifen för att förhindra mastcellsdegranulering och en sista grupp gavs PAF-receptorantagonisten WEB2086. HPE från *H. pylori*-stammen 88–23 applicerades på magslemhinnan sedan det övre löst fastsittande slemlagret tagits bort (se delarbetena I–III). I djuren som enbart förbehandlats med L-NNA sänkte HPE blodflödet med 19% efter 40 min och blodflödet fortsatte ner med ytterliggare 8%, under de efterföljande 20 min. Däremot, genom att förbehandla med ketotifen eller WEB2086 kunde effekten av HPE på blodflödet upphävas.

Dessa resultat föreslår att mekanismen bakom blodflödessänkningen med HPE beror på en frisättning av PAF från aktiverade mukosala mastceller. Enbart en inhibering av NO-produktionen räcker inte till för att sänka blodflödet i slemhinnan. Då NO normalt stabiliserar mastceller, kan det dock inte uteslutas att en bakteriell hämning av NO-produktionen skulle öka mastcellernas benägenhet att degranulera och frisätta inflammatoriska ämnen, skadliga för den omkringliggande vävnaden.

DISCUSSION

In this thesis, for the first time, the mucus gel layer was studied throughout the gastro-intestinal tract in an *in vivo* system. This offers the possibility of comparing different areas and relating the physical properties of their mucus gel layers to the function of the segments. In several other studies the effects of various noxious agents and bacterial products on the mucus gel layer have been studied. To date, no one has followed the increase in mucus thickness over time or changes in the renewal rate of the mucus gel layer under the influence of *H. pylori*.

The mucus gel layer — a continuous protective barrier!?

In visualizing the mucus gel layer as an effective physical barrier to the outer milieu, it seems obvious that it has to be continuous and of high quality. Yet, it has been questioned if the mucus layer really is a continuous layer or if it only covers the mucosa in a patchy manner. This question has arisen due to the findings in mucosal sections following histological fixation. Obviously, the thickness of the gel layer depends on the hydration of the mucins, which makes it sensitive to dehydration and drying procedures. In addition, the shear forces of washing the sections during preparation may erode the surface mucus layer. Previous *in vivo* studies, using the same technique in the stomach (Holm and Flemström, 1990, Schade *et al.,* 1994, Synnerstad and Holm, 1997) and in the duodenum (Sababi *et al.,* 1995), have supplied observations of a continuous mucus layer in these regions. The results in papers I and II support these observations and also supply evidence for a continuum of the mucus gel layer in the small intestine and the colon.

The mucus layer is a multi-layered structure

A novel discovery was made during an attempt to remove the mucus layer by careful suction. In the stomach and colon, in particular, there always remained a thick firmly adherent mucus layer which was impossible to remove by further suction (Papers I and II) or by using a moist cotton swab. Thus, it would seem that the mucus layer not only is continuous, but a thick layer always remains attached to the mucosa. The thickness of the firmly adherent layer was 80 µm in the corpus, 154 µm in the antrum and 116 µm in the colon (Paper I) with equivalent values in the stomach, obtained in paper II. In the small intestine, however, this mucus layer was less than 20 µm thick and had a patchy distribution with several villi completely free from mucus. The entire mucus layer in the small intestine can most likely be removed, although at the risk of damaging the villi.

These thickness values for the firmly adherent mucus layer in the stomach correspond fairly well with those obtained *in vitro* from unfixed mucosal sections, 80–120 µm (Kerss *et al.,* 1982) and 145 µm (Sandzén *et al.,* 1988). Presumably, the loosely adherent layer is largely lost during the washing procedures before processing. The same seems to be the case in a recent *in vivo* study using a confocal imaging system in which the corporal mucus thickness had a median value in the interval 50–75 µm, but 23% of the values were in the interval 0–25 µm (Chu *et al.,* 1999). In this case luminal perfusion of the mucosa may be part of the explanation for the thinner total mucus gel layer, although a reduction to 0–25 µm would require other factors as well. Again, in another *in vivo* system, studies of an inverted mucosa with a light microscope gave a mucus thickness value of 118 µm in the stomach of the rat (Kaunitz *et al.,* 1993). However, *in vitro* studies of inverted mucosa using the slit lamp and pachymeter (Bickel and Kauffman, 1981) and a recently modified histological method for cryostat sections provide total

mucus thickness values in the stomach close to those obtained in this study (Jordan *et al.,* 1998). The values obtained when using conventional histological techniques were much lower; over 50% thinner in the stomach (Jordan *et al.,* 1998), ≈80% thinner in the duodenum (Szentkuti and Lorenz, 1995) and ≈95% thinner in the colon (Rubinstein and Tirosh 1994, Matsuo *et al.,* 1997). Thus, the present study further emphasizes the loss and condensation of the mucus gel layer in earlier histological preparations.

Obviously, the earlier proposed organization of the mucus into three phases (Allen *et al.,* 1993, Allen and Carroll, 1985) will have to be modified. In keeping with the results from paper I the mucus layer should be divided into four phases: presecreted mucus stored in secretory granules in the mucus producing cells; the firmly adherent mucus layer closest to the mucosal surface; the loosely adherent mucus layer; and mobile (degraded and/or sloughed off) luminal mucus.

Why two distinct adherent mucus layers?

Shear forces, as mimicked by suction, would attend the digestive processes and therefore, the firmly adherent layer, particularly in the stomach and colon, would represent the mucus barrier normally intact during the digestive cycle. A general function of the loosely adherent mucus gel could be to provide lubrication for the mechanical propulsion of chyme down the gastrointestinal tract and hence, protect the integrity of the underlying firmly adherent layer and the mucosa. Another obvious function is related to the capacity of the mucus to bind pathogens (Forstner and Forstner, 1994) and larger molecules. Sloughing off the loosely adherent layer during the digestive process would subsequently remove these unwanted elements and prevent them from penetrating deeper down toward the mucosal surface. The thickest loosely adherent layers were found in the colon and ileum (Paper I). The chyme becomes more “solid” in the aboral direction, thereby requiring adequate lubrication, and the colon in particular harbors a large number of bacteria (Simon and Gorbach, 1987, Schultsz *et al.,* 1999).

A thicker stable unstirred mucus layer close to the epithelium might be necessary to support surface neutralization of back-diffusing acid and establishing a pH–gradient. Interestingly, the thickness of the firmly adherent layer in the present study was similar to the thickness of the pH–gradient (≈115 µm) found *in vivo*, with luminal pH2 during pentagastrin-stimulated acid secretion (Schade *et al.,* 1994). Acid secretory channels, recently demonstrated in the mucus gel layer, are most prominent in the firmly adherent layer (Johansson *et al.,* 2000), suggesting that it may be a prerequisite for their formation, thereby affording protection against mucosal acidification.

The apparent patchy appearance of the firmly adherent layer in the small intestine fits well with the major absorptive function of this region. Indeed, the mucus layer permits diffusion of smaller molecules but restricts diffusion of molecules as small as prostaglandins (Flemström *et al.,* 1999). The finding that the thickness of the firmly adherent layer did not mirror that of the total mucus thickness of the different regions (Fig. 5) would be in parity with the differing functions in these segments as discussed above. Ultimately, this suggests a difference in quality and physical properties between the two layers.

What is the difference between the two layers?

These experiments are yet to be performed and the answer can only be based on speculations. Findings in earlier studies of the gastrointestinal tract present a couple of plausible

explanations: gradual degradation/dilution, different mucins, different lipid contents or incorporated trefoil peptides.

Gradual degradation?

An obvious possibility is that the luminal portion of the mucus gel is gradually degraded by luminal enzymes as well as becoming more hydrated, thereby transferring to a weaker structure. The dominating secretion of the MUC2 mucin in the intestine (Allen *et al.,* 1998) embraces this explanation, as the two layers are less likely to consist of two different mucin populace. However, the colon undoubtedly has a thick firmly adherent mucus layer (Paper I). A recent *in vitro* study in the colon suggests that the colonic mucus layer does actually comprise of two distinct layers (Matsuo *et al.,* 1997). The thicknesses of the layers were approximately ten times thinner than observed in this study, but the stratification of the mucus layer was suggested to depend on differences in content of sulfated sialomucins. As such, the inner layer consisted of non–sulfated sialomucins and the outer layer of an alternating array of sulfated and non–sulfated sialomucins. This inner layer was, however, not found in the stomach or ileum, which confers that there might be differences in the structures of the mucus layers in the different regions.

Different mucin entities?

The finding of two different mucins in the gastric mucosa (Nordman *et al.,* 1998), MUC5AC in surface epithelial cells and MUC6 in the mucous neck cells (Ho *et al.,* 1995, Porchet *et al.,* 1995), has lent credence to another intriguing idea. A possible scenario is that the surface mucus cell mucin is the main constituent of the firmly adherent layer, while MUC6 is the main constituent of the loosely adherent layer. Indeed, a recent report describes a non–blended laminar arrangement of the gastric mucus gel, consisting of sheets of MUC5AC covering the mucosa and smaller amounts of MUC6 interspersed in non–continuous sheets in between (Ho *et al.,* 2000). This discovery is supported by earlier work with carbohydrate staining (Ota and Katsuyama, 1992, Ishihara and Hotta, 1993). Another recent report, suggests that *H. pylori* co–localizes with only MUC5AC (Van den Brink *et al.,* 2000), which would further support the idea that MUC5AC forms the juxtamucosal firmly adherent layer. In addition an aberrant expression of MUC6 by the surface epithelial cells resulting in a weakening of the firmly adherent gel during *H. pylori* infection (Byrd *et al.,* 1997), further defines possible structural differences between the gels formed by the two mucin products. This may not be a valid model in the intestine, as MUC2 is the predominant mucin (Allen *et al.,* 1998). However, the finding of two different mucus secretions from the goblet- and columnar cells of human colonic crypts (Halm and Troutman Halm, 2000), suggests that there might be a mixture of mucins, which contribute to the dual layered nature of the mucus gel in the colon.

Varying lipid content?

Lipids have been suggested to strengthen the mucus gel and protect it from luminal acid (Slomiany and Slomiany, 1991, Lichtenberger, 1995). The highest values have been found coating the stomach and colon, which would agree with the finding in this study of a firmly adherent layer in these regions. Certainly, the firmly adherent layer could contain a high amount of lipids but this may be a complement to the structural differences between the layers in terms of mucin content, and glycosylation and sulfation patterns.

Cross–linking trefoil peptides?

An exciting recent finding is that trefoil peptides co–localize with the mucins in the cell and in the gel and may be of importance for their secretion, gelation etc (Wong *et al.,* 1999, Newton *et*

al., 2000, Lichtenstein, 2000). Of particular interest is the co–localization of specific trefoil peptides with specific mucins, e.g. TFF1 with MUC6, TFF2 with MUC5AC and TFF3 with MUC2. Only TFF2 has two trefoil domains (homologous sequence of 42–43 amino acids) which are required for it to function as a cross–linker between mucin molecules. However, all trefoil peptides may dimerize and this form seems to offer better protection against noxious agents, and enables all trefoil peptides to cross–link mucins. Again, a possible explanation for the formation of a firmly adherent layer may be the content of specific trefoil peptides. It has been seen, that adding the trefoil peptide TFF2 to mucin *in vitro* reduces its permeation to protons (Tanaka *et al.*, 1997) — Is this of importance in the formation of a pH–gradient and possibly acid channels in the firmly adherent layer?

Mucus secretion and the effect of H. pylori

Intestine

There was a continuous mucus secretion in the intestine with the highest rate in the colon (Paper I). On mechanical stimulation by mild suction, the rate of increase in mucus thickness was not increased as might have been expected with reference to the compound exocytotic response characteristic for luminal irritants (Zalewsky and Moody, 1979, Specian and Oliver, 1991, Forstner and Forstner, 1994). The secreted mucus replenished the loosely adherent layer only as the thickness of the firmly adherent layer was unchanged on a second removal (Paper I). Although a reduced mucus barrier and accumulation rate would enhance uptake of nutrients, it is likely that mucus secretion would increase if required to protect the intestinal mucosa. Luminal acid was shown to increase mucus secretion in the duodenum after removal by suction (Sababi *et al.*, 1995).

Stomach

There was no measurable basal mucus secretion in the stomach (Paper I). This may be due to the lack of secretion or a low secretion rate balanced by luminal aggressors. Upon mucus removal there was a rapid burst of mucus which declined with time (Papers I and II). Unlike the case in the duodenum, this may have been a compound exocytotic response. Similar to the intestine, the secreted mucus formed a new loosely adherent layer since the thickness of the firmly adherent layer remained the same after a second mucus removal. In paper II, HPE's were administered acutely, immediately following mucus removal. The mucus renewal rate was significantly attenuated and again the firmly adherent layer was unaffected. Thus, it would seem that the acute effect of *H. pylori* would be to reduce the secretion of the loosely adherent layer. Surprisingly, the mucus renewal rate was similar in controls and in animals with a chronic *H. pylori* infection after mucus removal by suction (Paper II). However, the thickness of the firmly adherent and the loosely adherent layers were reduced in the corpus, while the thickness of the firmly adherent layer alone was reduced in the antrum. Thus, it seems that the bacterial effect on mucus secretion may change in the chronic phase of the infection or be partly compensated. Taken together, these results may explain the finding, in fixed sections, of a reduced mucus thickness in mice acutely given a *H. pylori* sonicate orally (Ghiara *et al.*, 1995). In addition, patients with chronic *H. pylori* infection have a reduced mucus gel layer thickness in the stomach (Sarosiek *et al.*, 1991).

Control animals exposed to luminal acid exhibited an even greater release of loosely adherent mucus in the antrum compared to that after removal by suction alone (Paper II). The response in the *H. pylori* infected animals remained the same as earlier, suggesting an effect on their

ability to respond to luminal acid in the antrum, while the response to shear stimulation persisted. A recent study demonstrated that the mucus released on luminal acid stimulation of the mucosa was solely derived from the mucous neck cells (Komuro *et al.,* 1992). Pilot experiments also suggest that the loosely adherent layer may be necessary to maintain the pH–gradient in the antrum (Atuma *et al.,* 1998). The loosely adherent layer thus appears necessary for the protection against luminal acid and is acutely attenuated by *H. pylori.* A study in patients with a chronic infection revealed that the juxtamucosal pH in the corpus was reduced, although not in the antrum (Frieri *et al.,* 1995). In conjuncture with the specific localization of mucins in the stomach (Ho *et al.,* 1995, Porchet *et al.,* 1995), *H. pylori* may inhibit the release of MUC6 from the mucous neck cells, which also fits in well with the higher concentration of bacteria normally found in the pit region. Of note is that the mucus layer, although thinner in *H. pylori* infected animals, still formed a continuous blanket covering the mucosa (Paper II).

H. pylori *reduces mucosal blood flow*

An adequate blood flow is vital for maintenance of mucosal integrity. However, the gastric microcirculation, like the mucus layer, seems to be a primary target for *H. pylori.* This has earlier been demonstrated in patients with a chronic infection (Lunde and Kvernebo, 1988) but has not yet been investigated if this may be an early event in *H. pylori* pathophysiology. The reduction in blood flow was seen in both the corpus and the antrum. In the present studies the corporal region was used to study the effects of *H. pylori* products on mucosal blood flow. Warm water extracts, prepared from two different type I strains of *H. pylori* (88–23 and A5), were applied luminally immediately after removing the loosely adherent layer in the corpus region of the stomach (Paper II). The mucus removal in itself induced a slight transient increase in blood flow, suggesting a mild mechanical stimulation compared to the more prolonged effects of tactile stimulation observed earlier (Holm and Jägare, 1993). The decrease in blood flow began immediately, later stabilizing at a level approximately 15% under basal control level (Table II). These results raise several questions to the nature of the mechanism behind the effect.

What factor is responsible for the reduction in blood flow?

The first question concerns the bacterial product behind the effect. A HPE from an isogenic mutant lacking production of the well-characterized virulence factors VacA and CagA produced the same results (Paper II). This is in support of the finding that the mucus layer restrains VacA (Flemström *et al.,* 1999). Hence, the early decrease in blood flow suggests that the mediator of the effect must be small enough to easily traverse the firmly adherent mucus gel layer. The factor could be common to several bacterial strains and therefore an extract from *E. Coli* was prepared. Luminal application induced no decrease in blood flow, suggesting that the factor may be specific for *H. pylori*. An attempt was made to attenuate the effect by boiling the HPE from 88–23. However, the same reduction in blood flow was attained, implying that it was heat stable. In contrast to this a boiled extract did not cause a reduction in mucus thickness in a mouse model (Ghiara *et al.,* 1995), and lost its pro–adhesive qualities in the mesenteric circulation (Yoshida *et al.,* 1993).

In a recent study by Craig *et al.* (1992), a heat stable and acid resistant factor with a molecular weight of 3000 was found in extracts from *H. pylori*. This factor possessed a chemotactic activity for monocytes and neutrophils *in vitro*. Indeed, an increase in neutrophil activity has been associated with *H. pylori* pathogenicity (Mooney *et al.,* 1991) and linked to microvascular dysfunction in the mesentery (Yoshida *et al.,* 1993, Kurose *et al.,* 1994). An alternative is the *H.*

pylori LPS, which is a heat stable, pro–inflammatory factor. LPS has priming effects on monocytes and neutrophils (Nielsen *et al.,* 1994, Perez–Perez *et al.,* 1995). However, LPS is present in *E. Coli* and is over 2000 times more potent (Perez–Perez *et al.,* 1995). The conclusion by Yoshida *et al.*. (1993), was that the effect probably was multifactorial possibly in part due to LPS.

Is the effect due to an inhibition of endogenous NO production?

The second question is to the mechanism behind the effect. Nitric oxide is a potent vasodilator involved in the endogenous regulation of gastric microvascular perfusion and inflammatory response, etc (Pique *et al.,* 1989, Wallace and Miller, 2000). Consequently, an inhibition of NO synthesis should result in a decrease in blood flow due to vascular constriction and increased peripheral resistance. In a recent study, a HPE was found to increase tissue concentrations of ADMA, an L–arginine analog that inhibited NOS in the duodenal mucosa (Fändriks *et al.,* 1997). Could the reduction in blood flow be the result of a bacterial inhibition of endogenous NO production? To test this hypothesis, L–NNA was used to pre–treat animals prior to mucus removal and application of HPE from 88–23 (Paper IV). In this way HPE would be prevented from further reducing NO production. L–NNA administration induced a transient increase in blood flow, which returned to control levels after approximately 50 min. Again, on application of the HPE blood flow was reduced. An interesting observation was that the reduction in blood flow did not stabilize but continued for the remainder of the experiment. The decrease in blood flow was also greater than that obtained earlier (Paper III). Thus, a reduction in NO production alone was not the causal mechanism behind the reduction in blood flow.

An inhibition of NOS has been reported to augment the endotoxin injury in the intestinal mucosa (Hutcheson *et al.,* 1990). In contrast, a recent study found that inhibition of iNOS ameliorated the endotoxin-induced mucosal barrier dysfunction (Unno *et al.,* 1997). The expression of iNOS in the gastric mucosa is actually increased in gastritis patients (Fu *et al.,* 1999). In other studies a NOS-inhibition in itself resulted in microvascular dysfunction in the mesentery (Kubes and Granger, 1992, Harris, 1997). In the present study NOS inhibition did not affect mucosal blood flow. A point to note, however, is that the effect of a NOS-inhibition on mucosal blood flow is also dependent on the anesthetic agent used (Holzer, 1994).

In a previous study in the mesentery, the effect of HPE could be divided into an early phase (after 10 min) and a late phase (after 30 min), both manifested by increased vascular protein leakage (Kurose *et al.,* 1994). Albumin leakage could cause interstitial edema possibly followed by vascular compression, and offers a plausible mechanism to the HPE reduction in blood flow. The later phase was also associated with increased leukocyte adhesion and emigration and the formation of leukocyte/platelet aggregates. NO normally modulates the leukocyte-endothelial cell interaction and leukocyte aggregation (Wallace and Miller, 2000). This suggests that the inhibition of NOS per se, may be the reason why mucosal blood flow continues to decrease in the L–NNA group after removal of the HPE. If this is the case, NO may normally be produced to counteract the late phase events of the HPE. These results do not rule out that a bacterial inhibition of NO may still be involved and possibly be a prerequisite for all or some of the actions of HPE.

Role of mast cell mediators

The mast cell stabilizer, ketotifen, completely attenuated the HPE mediated reduction in mucosal blood flow, as did the PAF receptor antagonist, WEB2086 (Paper IV). Thus, PAF

possibly derived from degranulating mast cells, was responsible for the HPE effect on the gastric microvasculature. Nitric oxide is an important regulator of mast cell reactivity (Mansini *et al.*, 1991, Wallace and Miller, 2000). Thus by concomitantly inhibiting NOS the ability to degranulate might be greater which may also explain the greater decrease in blood flow in the L–NNA group (Paper IV).

Mast cell mediators have been reported to mediate the effects of HPE superfusion in the mesentery (Kurose *et al.*, 1994) and an increased number of degranulated mast cells have also been seen in patients with gastritis (Nakajima *et al.*, 1997). The early phase vascular leakage observed in the mesentery could be attenuated by ketotifen and WEB2086 pre-treatment (Yoshida *et al.*, 1993, Kurose *et al.*, 1994). The late phase effect, however, was not affected and was suggested to depend on leukocyte-endothelium interactions and leukocyte/platelet aggregates. In keeping with this, the effect of HPE on gastric mucosal blood flow in these studies (Papers III and IV), may only be mediated by the microvascular actions of mast cell-derived PAF and independent of leukocyte–endothelium interactions. In a recent study, HPE-induced an early, transient, microvascular leakage in the stomach, which was attenuated by Ketotifen, but not by a PAF receptor antagonist (Kalia *et al.*, 2000). Moreover, the HPE application induced a PAF-dependent aggregation of platelets in the microvasculature, which could not be blocked by ketotifen. Blood flow was not measured and it is impossible to say if the tissue edema or the aggregation of platelets may reduce blood flow per se or if both components are required, since the observed vascular leakage was transient.

Taken together, these results suggest that PAF, derived from mast cells or possibly from *H. pylori* (Denizot *et al.*, 1990), is involved in the acute effects of *H. pylori* on the gastric microvasculature. Indeed, PAF has been recognized as the mediator of endotoxin induced injury in the gastrointestinal tract (Wallace *et al.*, 1987). The reduction in blood flow in the present studies (Papers III and IV) by 15–27% was less than the 40% suggested to be a threshold level for increased acid induced injury (Leung *et al.*, 1985). It is possible, however, that the acute reduction in blood flow is followed by leukocyte infiltration and a chronic inflammatory condition with further deleterious effects on the gastric microvasculature.

Acid secretion

Changes in acid secretion have been conferred a large role in the development of gastroduodenal ulcers. The effects and importance of *H. pylori* on acid secretion are not fully resolved, but an acute hyposecretion followed by a more moderate to increased secretion has been suggested (Calam, 1995, McGowan *et al.*, 1996). No definite conclusions can be drawn based on the disperse results on acid secretion in the present acute studies; HPE from A5 and A5VacA increased acid secretion, while HPE from 88–23 had no effect (Papers II and III). This may be because the corpus or antrum were studied separately in these experiments and as such any effect on acid secretion, due to the actions of HPE in the antrum, could not be measured. However, in the chronically infected animals acid secretion was normal and similar to that in controls (paper II).

A decreased acid secretion, with facilitated *H. pylori* colonization of the stomach, has been suggested as a cause and result of atrophic gastritis with subsequent progression to cancer (Blaser, 1992, Kuipers *et al.*, 1995, Genta, 1997). An increase in acid secretion would imply a greater acid load in the duodenum, causing the formation of gastric metaplastic foci in which the epithelial cell phenotype is similar to that in the stomach (Walker and Dixon, 1996). *H. pylori* readily colonizes these metaplasia, which ultimately may be a prerequisite for the development of duodenal ulcers. Acid secretion is regulated by gastrin secreted from G–cells

in the antrum and *H. pylori* is thought to increase gastrin release, the so called “gastrin link”. *H. pylori* concomitantly hampers the somatostatin-mediated inhibition of acid secretion (Olbe *et al.*, 1996, Sawada and Dickinson, 1997, Calam *et al.*, 1997).

SUMMARY AND CONCLUSIONS

A continuous mucus gel layer forms a protective blanket over the gastrointestinal mucosa from the stomach to the colon. The mucus gel is a multi-layered structure with a lower firmly adherent layer and a loosely adherent upper layer, that can easily be removed by mild shear. The relative thickness of the two layers of the mucus gel varies for different regions of the gut. Following mucus removal a rapid renewal of loosely adherent mucus ensues. The loosely adherent layer is in keeping with an essential and expendable lubricant continuously replaced on mechanical stimulation. A firmly adherent more resistant layer would be essential for the barrier functions and protection against luminal aggressors. The patchy distribution of the firmly adherent layer in the small intestine may reflect its absorptive function, which requires that the luminal contents come into contact with the mucosal surface.

In the stomach a rapid renewal of the mucus layer ensued upon removal of the loosely adherent layer. The increase in renewal rate was furthered by luminal acid in the antrum. Water-soluble factors from *H. pylori* acutely attenuated the basal mucus renewal, while a chronic infection with *H. pylori* only attenuated the response to luminal acid. In addition, the mucus gel layers were considerably thinner during a chronic infection. Thus, an acute reduction in mucus release and thereby, mucus thickness, may be necessary to ease colonization. The attenuation of mucus release on acid stimulation and a concomitant thinner mucus gel layer may be one causative factor to the development of mucosal injury during a chronic *H. pylori* infection.

An adequate blood flow in the stomach is essential for its normal function and protection against luminal aggressors. Acute application of water extracts from *H. pylori* reduced mucosal blood flow by approximately 15%. This reduction in itself may not be enough to increase the mucosal susceptibility to injurious agents. However, a reduced mucosal perfusion in combination with a decreased thickness of the firmly adherent mucus layer and an attenuated mucus secretory response to luminal acid, would markedly compromise mucosal protection.

Mucosal mast cells act as alarm cells reacting on luminal antigens and initiating an inflammatory response. Nitric oxide modulates the activity of the mast cells and also maintains adequate perfusion in the tissue. *H. pylori* products reduce mucosal blood flow independent of NO production, by causing a release of PAF probably from degranulating mast cells. Hence, the inflammatory response to *H. pylori* in itself increases the mucosal vulnerability and possibly enhances bacterial access to the mucosa. The reduction in blood flow was possibly augmented by inhibiting NO production, suggesting that endogenous NO may be produced to counteract the effects of the bacteria.

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Figura N. Identifiable *Helicobacter pylori* strains or factors important in the development of duodenal ulcer disease. *Helicobacter 1997;2 suppl 1:S3–12.*

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