Impaired mucus-bicarbonate barrier in *Helicobacter pylori*-infected mice

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Short title: *H. pylori* impairs the mucus-bicarbonate barrier

Abbreviations

- α-1,3/4FT transgenic mouse expressing human α-1,3/4 fucosyltransferase
- CFU colony-forming units
- i.p. intraperitoneal
- LDF laser-Doppler flowmetry
- Le\(^b\) Lewis\(^b\) histoblood group antigen
- MAP mean arterial pressure
- NOS nitric oxide synthase
- PBS phosphate-buffered saline
- PFU perfusion units
- SLC26A9 solute carrier 26A9

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ABSTRACT

To resist the harsh intrinsic milieu, several lines of defense exist in the stomach. The aim of this study was to investigate the effect of the gastric pathogen *Helicobacter pylori* on these mechanisms in vivo. We used FVB/N mice expressing human α-1,3/4-fucosyl transferase (producing Le\(^b\) epitopes), inoculated with *H. pylori* 1. The mice were anesthetized with isoflurane or Hypnorm/midazolam and the stomach was exteriorized and the surface of the corpus mucosa exposed. Mucus thickness was measured with micropipettes, juxtamucosal pH (pH\(_{jm}\)) with pH-sensitive microelectrodes, blood flow with laser-Doppler flowmetry, and mRNA levels of the bicarbonate transporter SLC26A9 quantified with real time PCR. The increase in mucosal blood flow seen in response to luminal acid (pH 1.5) in control animals (140±9% of control) was abolished in infected mice. The firmly adherent mucus layer was significantly thinner in infected (31±2 μm) than in control mice (46±5 μm) and no mucus accumulation occurred in the infected mice. Juxtamucosal pH decreased significantly more on exposure to luminal acid in infected mice (luminal pH 1.5 pH\(_{jm}\) 2.4±0.7) than in control mice (pH\(_{jm}\) 6.4±0.5). In spite of a reduced pH\(_{jm}\) SLC26A9 mRNA expression was significantly increased 1.9-fold in infected mice. The reduction in pH\(_{jm}\) by infection with *H. pylori* might be due to a reduced firmly adherent mucus layer, increased mucus permeability to H\(^+\) and/or inhibition of bicarbonate transport. The upregulation of SLC26A9 in *H. pylori* infected epithelium might be a result of continuous inhibition of the transporter by e.g. ammonium, a *H. pylori* product, earlier shown to inhibit SLC26A9.

Keywords

Juxtamucosal pH, blood flow, laser-Doppler flowmetry, permeability,
INTRODUCTION

The regular exposure of the stomach to endogenously produced acid and degrading enzymes requires the presence of an efficient gastric mucosal barrier. The acidic milieu in the stomach is lethal to most bacteria, but Helicobacter pylori not only survives but actually colonizes the stomach in approximately 50% of the human population (15). A very important factor for H. pylori colonization is its cytoplasmic urease activity, which will produce ammonia from urea and is thereby able to handle the very high proton concentration in the stomach (8). H. pylori causes gastritis and is also considered to be a major ulcerogenic factor (22), as well as a carcinogen (18). The mechanisms underlying the development of these pathologic changes are still not clear, but disruption of the mucosal barrier is most certainly involved. The only natural host for H. pylori are primates and humans (23) and it is therefore difficult to infect commonly used laboratory animals such as rats and mice. In this study, however, a transgenic mouse that use human α-1,3/4-fucosyl transferase (α-1,3/4FT) to express Lewis b epitopes in the surface epithelium was used (10). Lewis b mediates attachment of H. pylori and it has been demonstrated that H. pylori establishes a chronic, high-density infection in this line of mice (10).

The gastric mucosal barrier consists of a pre-epithelial mucus-bicarbonate barrier, a tight epithelial barrier, and a subepithelial component including blood flow and nerves. The pre-epithelial layer involves both secreted mucus and bicarbonate. Next to the epithelial cells we have identified a firmly adherent mucus layer which is impossible to remove by suction or by wiping with a cotton tip. Luminally to this layer there is yet another adherent layer, which, however, is removable, and this is designated the loosely adherent layer. In previous studies in rats we have shown that the pH at the epithelial surface (juxtamucosal pH, pHjm) is neutral despite an acidic lumen (28, 35). Only the firmly adherent layer is important in maintaining
the juxtamucosal pH at a neutral level during luminal acid exposure (28). Thus, the firmly adherent layer of mucus together with bicarbonate secreted from the surface epithelial cells seems to form a pH gradient with a neutral pH next to the epithelial cells even though the luminal pH is acidic (28, 35).

SLC26A9, a cloned member of SLC26 family of anion exchangers was recently shown to mediate Cl⁻/HCO₃⁻ exchange and was found in the apical membrane of gastric surface epithelial cells (39). In addition to Cl⁻/HCO₃⁻ exchange, SLC26A9 can function as a chloride-independent bicarbonate transporter. Thus, given its localization and functional modes, SLC26A9 may be important in creating and maintaining neutrality of the juxtamucosal pH. The SLC26A9-mediated Cl⁻/HCO₃⁻ exchanger was shown to be inhibited by NH₄⁺ (39). This is in contrast with SLC26A3, expressed in colonic epithelium, which is actually stimulated by NH₄⁺ (6). Recent studies from our laboratories demonstrated that the gastric lumen of rats treated with NH₄⁺ could not maintain a neutral juxtamucosal pH when challenged with acid (39). Given the abundant amount of ammonium/ammonia that is produced by *H. pylori* urease activity it was suggested that *H. pylori* can disrupt the mucus-bicarbonate layer by inhibiting SLC26A9-mediated HCO₃⁻ secretion (39).

Gastric mucosal blood flow has a vital role in gastric mucosal protection. It maintains the juxtamucosal bicarbonate gradient during acid secretion by transporting bicarbonate from the parietal cells to the surface epithelial cells, through which bicarbonate is secreted into the mucus gel layer. In addition an adequate blood flow is required to maintain the different functions of the mucosal cells, including the process of rapid repair. The blood flow is also important in removing back-diffusing hydrogen ions, toxic waste products, accumulated oxygen metabolites, and other inflammatory mediators (17). We have shown in previous
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studies that luminal acid alone induces hyperemia without any macroscopic lesions (29, 35). Furthermore, we suggested that epithelial inducible nitric oxide synthase (iNOS) is involved in the hyperemic response to acid, possibly signaling to afferent nerves, leading to a blood flow increase.

The purpose of this study was to investigate how a chronic infection with *H. pylori* influences the gastric mucus thickness and accumulation, the juxtamucosal pH, the gastric mucosal blood flow response to acid, and the expression of the Cl⁻/HCO₃⁻ exchanger SLC26A9; all of which are important factors in the gastric protection barrier.
MATERIALS AND METHODS

All experimental procedures in this study were approved by the Swedish Laboratory Animal Ethical Committee in Uppsala and were conducted in accordance with guidelines of the Swedish National Board for Laboratory Animals.

Mice

FVB/N mice expressing human α-1,3/4FT were used. These mice produce Le^b epitopes in the surface epithelium, as previously described by Falk et al. (10). Breeding pairs of mice expressing the epitope were kindly provided by Per Falk (Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO, USA). The genotype of each mouse was determined by PCR analysis of DNA isolated from tail tissue. Mice expressing α-1,3/4FT were used for both the control and infected group. 34 female and 21 male mice, weighing between 22 and 43 g, were used for the experiments (equally distributed in the experiments).

Infection

The strain Hp1, isolated from a Peruvian patient with gastritis, was used. The strain has been shown to infect this line of mice under normal conditions (14). Isolates of Hp1 were cultured on Columbia II agar plates (BBL, Becton Dickinson and Company, Cockeysville, MD) with 10% horse serum and 8.5% denatured horse blood (chocolate agar) under microaerophilic conditions at 37°C. The bacteria were suspended in sterile phosphate buffered saline (PBS) solution to a concentration of \(10^7\)-\(10^8\) colony-forming units (CFU)/ml. Organisms were inoculated into the oral cavity with a pipette (50 µl) twice, or by gavage (200 µl) 4 times during a two-week period. Control animals were treated with PBS only. The mice were deprived of food and water for 2 hours prior to each inoculation. The animals were between 4
and 12 weeks old at the time of inoculation and the infection was allowed to stabilize for 11 to 21 weeks (average 15±1 week).

After the experiment, one half of the stomach was homogenized in 1 ml of Helicobacter broth (Uppsala University Hospital, Sweden), and 100 µl of the suspension was plated on selective medium (Blood Agarbase 2 CM271 Oxoid, 7% horse blood, Skirrow SR69 Oxoid supplement). Single colonies were picked up and recultured on chocolate agar plates. Isolates were identified as *H. pylori* on the basis of their morphology (Gram-staining) and their presence of urease activity. Animals not confirmed positive were excluded. In some of the experiments the colonies were counted and expressed as CFU/mg stomach. To evaluate the degree of inflammation, infected (n=3) and control (n=3) mice were decapitated and one half of the stomach was fixed in paraformaldehyde 4% and embedded in paraffin. Sections 3 µm thick were prepared and stained with hematoxylin and eosin, following standard procedures. The inflammatory cell infiltration was graded blindly.

**Anesthesia**

The animals were anesthetized with either isoflurane (for mucus measurements, blood flow measurements, and mucosal permeability measurements) or hypnorm/midazolam (for pH_{imb} measurements). Isoflurane anesthesia was induced by spontaneous inhalation (Forene®, Abbott Scandinavia AB, Kista, Sweden). The inhalation gas was a mixture of air and oxygen (total oxygen 40%) and ≈2.4% isoflurane. The Hypnorm/midazolam anesthesia was induced with an intraperitoneal (i.p.) injection of Hypnorm (0.4 ml kg^{-1}, giving 0.126 mg kg^{-1} fentanyl citrate and 4 mg kg^{-1} fluanisone) and midazolam (5 mg kg^{-1}) mixed together in a volume of 0.2-0.3 ml. The anesthesia was maintained by a continuous intraarterial infusion of Hypnorm in a modified Ringer solution and in a few experiments in 2% albumin, given at a
concentration that kept the mice at an anesthetic level. Additional midazolam was given i.p. when required.

**Animal preparation**

Body temperature was maintained at 37°C by means of a heating pad controlled by a rectal thermistor probe. A catheter containing heparin (12.5 IU ml⁻¹) dissolved in isotonic saline was placed in the carotid artery to monitor blood pressure. The jugular vein was cannulated for continuous infusion of a maintenance fluid (modified Ringer solution, 0.35 ml/h). In a few experiments the vein cannulation failed and the infusion was performed via the arterial catheter through a Y connection.

The preparation of the gastric mucosa for intravital microscopy in mice has been described previously (16). Briefly, exteriorization of the mucosa through a midline abdominal incision was followed by an incision along the greater curvature in the forestomach. The animal was placed on a Lucite table with part of the corpus of the stomach loosely draped over a truncated cone, with the mucosal surface facing upwards. A "mucosal chamber" was fitted over the gastric mucosa, exposing approximately 0.13 cm² of the mucosa. The chamber was filled with 3 ml of unbuffered 0.9% saline, maintained at 37°C by means of warm water circulating in a jacket in the bottom of the chamber. The saline was replaced at regular intervals of 10-15 min and the pH of the solution was measured. The mouse was moved to a stereomicroscope (Leica MZ12; Leica, Heerbrugg, Switzerland) and the gastric mucosa was transilluminated. Before the experiments, the animals were allowed to stabilize for 20-60 min after surgery.
Mucosal permeability measurements

To investigate the effect of *H. pylori* infection on the gastric mucosal integrity, the mucosal permeability was determined by measuring the clearance of $^{51}$Cr-chromium-labeled EDTA ($^{51}$Cr-EDTA, PerkinElmer Life Sciences, Boston, MA) from blood to lumen (26).

Fifteen minutes after completion of surgery $^{51}$Cr-EDTA (5-7.5 μCi) was injected as a bolus dose (0.03-0.05 ml), followed by a continuous intravenous infusion of $^{51}$Cr-EDTA (15-60 μCi/ml in the Ringer solution) at a rate of 0.35 ml/h. One blood sample (~25 μl) was taken 30 min after the injection of $^{51}$Cr-EDTA, and a second one was taken 30 min later. After the first blood sample withdrawal, the blood volume loss was compensated for by injection of 7% bovine albumin in saline (0.05 ml). The luminal solution and the blood were analyzed for $^{51}$Cr activity in a gamma counter (model 1282, Compugamma CS, PerkinElmer Life Science, Wallac, Upplands Väsby, Sweden). The clearance value was calculated by dividing each individual effluent cpm value by a corresponding plasma cpm value and expressed as ml min$^{-1}$ 100g$^{-1}$ wet tissue weight.

Mucus measurements

Micropipettes made of glass tubing (borosilicate tubing, OD 1.2 mm, ID 0.6 mm; Frederick Haer&Co., Brunswick, ME), pulled to a tip diameter of 1-2 μm, and prepared as described earlier (21), were used. The mucus gel thickness was measured by inserting a micropipette from the luminal solution into the gel at an angle of 33°-39° to the surface, using a micromanipulator (Leitz, Wetzlar, Germany). The mucus thickness was measured by advancement and withdrawal of the tip of the micropipette at five different sites on the mucosa. The mean value of these five measurements was regarded as one measurement. A “digimatic indicator” (IDC Series 543; Mituoyo Corp., Tokyo, Japan) was connected to the
micromanipulator for measurement of the distance covered by the micropipette. The distance in the vertical direction (90° to the surface) from the luminal surface of the mucus gel was calculated.

After the measurements of the total mucus thickness, the loosely adherent layer was removed by gentle suction, allowing the thickness of the firmly adherent layer to be measured (first removal). Fifteen and 30 minutes later, the mucus thickness was again measured, whereby the rate of mucus accumulation could be calculated. Finally, the loosely adherent layer was removed again, and the thickness of the firmly adherent layer was measured once more (second removal).

**pH measurements**

The hydrogen ion concentration in the mucus gel at the epithelial cell surface, i.e. the juxtamucosal pH (pH\textsubscript{jm}), was measured with hydrogen ion-selective microelectrodes as described before (28). Glass tubing (borosilicate tubing with omega dot, OD 1.2 mm, ID 0.9 mm; Frederik Haer&Co, Brunswick, ME) was pulled to a tip diameter of 1-3 µm and filled up to a distance of ~300 µm from the tip with a proton cocktail (hydrogen ion Ionophore II-Cocktail; Fluka, Buchs, Switzerland). The remaining part of the electrode was filled with HEPES buffer at pH 7.4, connected by an Ag-AgCl wire to a dual differential electrometer with a high input impedance (FD223; Biomedical Center, Uppsala, Sweden). A reference electrode was placed in the saline covering the gastric mucosa. The electrodes were calibrated before and after the experiments in iso-osmolar (310 mOsm) solutions with a pH of 1.5-8 at 37°C. The microelectrodes were inserted into the mucus gel at an angle of 30-40° to the mucosa and placed at the mucosal surface, by means of a micromanipulator (Leitz, Wetzlar, Germany).
Since earlier studies in the rat had revealed that pH$_{jm}$ was not influenced by the loosely adherent mucus layer (28), and since the position of the electrode was easier to verify if the loosely adherent mucus layer was removed, this was done before the experiments were started. Ten minutes after the electrode had been placed in position, acid (HCl, pH 2) was instilled into the lumen for 10 min, after which it was changed to saline for 10 min, then acid (HCl, pH 1.5) for another 10 min, followed by 10 min of saline.

**Blood flow measurements**

Blood flow was measured with laser-Doppler flowmetry (LDF) equipment (Periflux instruments Pf3 and Pf4001, Perimed, Stockholm, Sweden) which had previously been used to study the microcirculatory blood flow of the gastric mucosa in the mouse model (16). The laser probe was held in a fixed position in the chamber solution at a distance of 1-2 mm above the mucosa by a micromanipulator. With the type and position of the probe used in these studies, the laser light most likely penetrates through the entire thickness of the gastric wall (20). However, the recorded blood flow is mainly mucosal, since the amount of back-scattered light decreases exponentially with the depth in the tissue.

The continuously measured blood flow is reported as percent of that in the control period, i.e., the 10 min period, prior to HCl application. After the stabilization period the loosely adherent mucus was removed, and the blood flow was recorded for 30 min, after which the same protocol as for pH$_{jm}$ measurements was used.
**SLC26A9 expression**

*Surface epithelial cells*

Two thirds of the stomach from Hp1 infected and control mice was gently scraped with the blunted end of a scalpel to obtain epithelial cells. The removed material was snap frozen in liquid nitrogen. To confirm that only the uppermost part of the mucosa was removed, the remaining tissue was fixed and stained as described above. The remaining intact 1/3 of the stomach was used for confirmation of infection, also described above.

*mRNA expression (Real-time PCR)*

Total RNA was isolated with TRIzol® (Invitrogen, Carlsbad, CA) from surface epithelial cell preparation of 5 control and 3 Hp1 infected animals. cDNA was synthesized using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) with random hexamers according to the protocol from the manufacturer.

A Rotor-Gene 3000 (Corbette Research, Australia), and detection with SYBR Green, was used to amplify and analyze generated cDNA. The primers used (Genebank accession # included) were glucose-6-phosphate dehydrogenase (G6PDH, #BC075663), 5’-ATTGACCACTACCTGGGCAA-3’, 5’-CCCTGATGATCCCAAATTCA-3’; SLC26A9 (#AK086815) 5’-TCGTTCTTCCTGAGCCTGCC-3’, 5’-CCAGTGTGGAGCCATTTCGA-3’.

PCR amplifications were performed in a total volume of 25.5 µl, containing 8 µl of cDNA, 0.3 µM of each primer, and 12.5 µl SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK). For each reaction, the polymerase was activated by a preincubation for 15 min at 95°C. Cycling parameters were 95°C, 20 s; 59°C, 30 s; and 72 °C, 40 s. The results are presented as threshold cycle values ($C_T$ values), i.e., the estimated amplification cycle
number when the fluorescence exceeds a specified threshold value. All samples were run in duplicate. The $C_T$ values were used to calculate the amount of PCR product in comparison to G6PDH as a "house-keeping gene", by subtracting the $C_T$ value for G6PDH from the $C_T$ value for SLC26A9 ($\Delta C_T$).

Semiquantitative immunoblotting

A total cellular fraction containing plasma membrane and intracellular membrane vesicles was prepared from pooled gastric surface epithelial cell preparations from 3 control and 3 Hp1 infected animals. The tissue samples were homogenized in ice-cold isolation solution (250 mM sucrose and 10 mM triethanolamine, pH 7.6) containing protease inhibitors (phenylmethanesulfonyl fluoride, 0.1 mg/ml; leupeptin 1 µg/ml), using a Polytron® System PT 1200 CL (Kinematica AG, Switzerland). The homogenate was centrifuged at low speed (1,000 x g) for 10 min at 4°C to remove nuclei and cell debris. The supernatant was centrifuged at 150,000 x g for 90 min at 4°C. The pellet containing plasma membrane and intracellular vesicles was suspended in isolation solution with protease inhibitors. The membrane fractions were solubilized at 65°C for 20 min in 5x Laemmli buffer (0.125 M Tris, 0.86 M Glycine, 1% SDS) and the total protein concentration was measured with DC Protein Assay (Bio-Rad, Hercules, CA). One hundred micrograms of total protein per well was loaded on 10% polyacrylamide minigels (Ready Gel® Precast Gels, Bio-Rad, Hercules, CA), size fractionated under denaturing conditions, and then transferred to a PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 8% milk proteins and then probed with affinity-purified anti-SLC26A9 immune serum. A goat anti-rabbit IgG antibody coupled to horseradish peroxidase (Bio-Rad, Hercules, CA) was used as a secondary antibody. Proteins were visualized by enhanced chemiluminescence detection (ECL+, Amersham
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Biosciences, Uppsala, Sweden). SLC26A9 positive bands were quantified with Quantity One software (Bio-Rad, Hercules, CA).

**Chemicals**

Chemicals used were: heparin (Leo Pharma AB, Sweden); Hypnorm® (fentanyl citrate 0.315 mg ml⁻¹ and fluanisone 10 mg ml⁻¹, Janssen Pharmaceutica, Beerse, Belgium); midazolam (Dormicum®, 5 mg ml⁻¹, Roche AB, Stockholm, Sweden); silicone grease (Dow Corning high vacuum grease, Dow Corning GmbH, Weisbaden, Germany); HEPES (Merck, Darmstadt, Germany); tributylchlorosilane (Fluka Chemie GmbH, Buchs, Switzerland); and bovine albumin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). DNA was purified and amplified using DNeasy Tissue Kit and Master Mix (Qiagen GmbH, Hilden, Germany). Modified Ringer solution: 120 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, and 0.75 mM CaCl₂.

**Statistics**

All values are presented as mean±standard error of the mean. Statistical significance was determined with student’s t-test or ANOVA for repeated measurements, followed by Fisher’s protected least significant difference test. The level of significance was set at p<0.05. Statview-SE and Graphics software (Abacus concepts, Inc., Berkeley, CA) were used for all statistical calculations.
RESULTS

Infection

No *H. pylori* was found in the non-inoculated mice, but these bacteria were observed in 90% of the *H. pylori* inoculated mice. The mouse body weight to stomach wet weight ratio did not differ between groups (control mice 135±8, n=8; infected mice 130±6, n=12). The density of the bacteria was 3.8±0.1 log CFU/stomach (n=12). With light microscopy, mild to moderate inflammation was noted in the *H. pylori*-infected stomachs in comparison with that in uninfected controls. No ulcers were detected but a small mucosal bleeding was observed in one *H. pylori*-infected animal after acidic challenge. The gastric permeability (51Cr-EDTA clearance) was significantly higher in infected mice than in control mice (Figure 1). We did not observe any gender differences in any of the parameters investigated.

Mucus measurements

Results from the mucus measurements are presented in Figures 2 A and B. The total mucus thickness (both loosely and firmly adherent) did not differ between the controls (n=5) and the infected (n=6) animals. However, when the loosely adherent layer was removed by gentle suction a significantly thinner firmly adherent layer was revealed in the infected animals. Thirty minutes after the first mucus removal, the mucus thickness had increased significantly in the control animals, and the mucus accumulation was calculated to be 9±3 μm or 0.3±0.1 μm min⁻¹. In the infected animals no significant mucus accumulation had occurred (3±1 μm or 0.1±0.04 μm min⁻¹). The mean arterial blood pressure decreased significantly during the experiments in both groups (from 71±5 to 63±3 mmHg in controls and from 75±5 to 67±5 mmHg in infected mice), with no difference between the groups.
**pH measurements**

Figure 3 shows the mean values for pH$_{jm}$ in control (n=7) and infected (n=5) animals. The juxtamucosal pH was neutral during the control period in both groups. When HCl pH 2 and 1.5 was applied luminally in the control animals, pH$_{jm}$ decreased significantly to 6.6±0.4 (transiently) and 6.3±0.6, respectively. In the infected animals, pH$_{jm}$ decreased significantly to 4.7±0.8 during topical administration of HCl pH 2 and returned to the control level after removal of the acid. During luminal instillation of HCl, pH 1.5, pH$_{jm}$ decreased continually to 2.4±0.7, and did not return to the control level until 10 minutes after removal of the acid. There was a significantly greater decline in pH$_{jm}$ in the infected animals than in the controls during luminal application of HCl both at pH 2 and 1.5. The mean arterial blood pressure decreased significantly during the experiments in both groups (from 67±3 to 61±3 mmHg in controls, and from 61±2 to 55±2 mmHg in infected mice). Acid secretion was negligible in both groups.

**Blood flow measurements**

Figure 4 presents results for gastric mucosal blood flow, vascular resistance, and blood pressure in control (n=6) and *H. pylori*-infected (n=6) mice during luminal application of acid. In control mice the blood flow increased significantly during luminal application of HCl, pH 2 and 1.5. This increase occurred despite a reduction in blood pressure. Accordingly there was a decrease in gastric vascular resistance. In infected animals, neither gastric mucosal blood flow nor blood pressure changed during the experiment. The baseline blood flow value expressed in perfusion units (PFU) per gram stomach weight was 301±92 in the controls and 292±43 in the infected animals.
SLC26A9 expression

The procedure of scraping the mucosa removed only the uppermost part of the mucosa, as revealed histologically (Figure 5C). Expression of SLC26A9 mRNA in surface epithelial cells from *H. pylori*-infected mice increased significantly 1.9-fold when measured by real time PCR. The ΔC_T values are presented in Figure 5A (control, n=5; infected, n=3). The results of semiquantitative western blot analysis of SLC26A9 showed the upregulation of the expected ~80 kDa band by ~4 fold in the *H. pylori* infected animals (Figure 5B, control, n=3; infected, n=3).
DISCUSSION

The mucus-bicarbonate layer is an important protective mechanism in the stomach and disruption of this barrier might be the way in which *H. pylori* induces pathological changes in the stomach. In this study we have shown that a chronic infection with *H. pylori* affects major mechanisms of gastric mucosal protection. In the presence of such infection the mucus layer is thinner, the mucus pH at the surface of the epithelial cells is more acidic when the luminal pH is reduced, and blood flow regulation is altered.

Here we have used a special mouse model, α1,3/4FT expressing mice, infected with a human *H. pylori* strain (Hp1). The α1,3/4FT mice use human α1,3/4-fucosyltransferase to express Lewis^b^ (Le^b^) in their pit region and surface mucus cells (14). Le^b^ is a blood group antigen which is recognized by an *H. pylori* adhesin, blood-group antigen-binding adhesin (BabA) (19). Thus, presence of Le^b^ in the host and BabA in the bacteria mediates adherence to the gastric epithelial lining and implies a supposedly more severe infection. Indeed, the α1,3/4FT mice develop more severe gastritis when infected than their nontransgenic littermates and also develop autoantibodies against parietal cells (14). Gastric permeability was also increased in the infected mice, in conformity with findings in *H. pylori*-infected patients (11) and animals (34).

A continuous mucus layer covers the mucosa of the stomach. We have previously shown in a rat model (3) and in this mouse model (16) that the mucus layer can be divided into two layers, one that can be removed (designated loosely adherent) and one that cannot be removed by mechanical means without destroying the epithelial layer (the firmly adherent layer). From another study, we have also concluded that it is the firmly adherent layer that is important for the protection against luminal acid, as it is in this layer that the pH gradient is
formed (28). In our unique mouse model in vivo we can measure the thickness of both the loosely and firmly adherent mucus layers, and also follow the mucus accumulation over time. Using this model we observed here that, when the loosely adherent mucus was removed, the firmly adherent layer in the infected mice was significantly thinner than in the non-infected controls, but still a continuous layer. Furthermore, no mucus accumulation was detected during the 30-minute measuring period. Clearly, mucus does accumulate also in the infected animals as we found the same total mucus thickness in both infected and control animals before the loosely adherent mucus was removed the first time. The accumulation time might however be longer in the infected situation.

Mucus accumulation is a result of synthesis, secretion, degradation and the quality of the mucus. Earlier in vitro and histology studies have shown that *H. pylori* might reduce the mucus gel thickness in different ways; for example mucin synthesis might be inhibited (4) mucin secretion reduced (36) or the mucins degraded (24). Furthermore, the expression pattern of the gastric mucins MUC5AC and MUC6 seems to be altered in *H. pylori*-infected patients and skewed toward more MUC6 (5, 37). Measurement of mucus thickness in biopsies, have shown both a decreased mucus thickness in *H. pylori* positive subjects (27, 31) as well as no difference in mucus thickness between *H. pylori*-positive and -negative subjects (1, 2, 24, 25). The present study is the only study presenting mucus thickness values from in vivo measurements including accumulation rate. We found that the total mucus thickness (loosely + firmly adherent mucus) did not differ between infected and non-infected animals. However, we have shown here that the important protective firmly adherent layer is thinner in *H. pylori*-infected animals and that accumulation of the loosely adherent mucus is delayed.
In the mucus layer, secreted bicarbonate neutralizes back-diffused acid and a pH gradient is formed, with a neutral pH adjacent to the epithelial cells (30, 32). This was also shown to occur in the control mice in the present study. However, the infected mice could not preserve a neutral juxtamucosal pH in the presence of luminal acid. \textit{H. pylori} could diminish the ability to maintain this neutral environment by several mechanisms. As we have shown in this study, these bacteria reduce the thickness of the firmly adherent layer. This layer had previously been shown, as discussed above, to be involved in maintaining a pH gradient. The mucus thickness per se or the properties of the mucus could be important in maintaining the unstirred layer that is required for maintenance of the pH gradient. In vitro studies have suggested that presence of NH$_4^+$, which is produced by \textit{H. pylori} urea splitting enzyme urease, could increase the permeability of protons in the mucus (7).

Another possibility is that \textit{H. pylori} inhibits bicarbonate transport across the epithelial cells or the expression of bicarbonate transporters. In this study, however, we observed increased mRNA expression and protein abundance of an important Cl/HCO$_3^-$ exchanger SLC26A9 in gastric surface epithelial cells in \textit{H. pylori}-infected mice. Based on this observation alone an obvious conclusion would have been that bicarbonate transport would increase. However, since the in vivo measurements showed a reduced pH at the surface of the epithelial cells, an inhibition of the bicarbonate transport most probable have occurred. Interestingly, we have shown in in vitro studies that SLC26A9 is inhibited by the \textit{H. pylori} product NH$_4^+$ (39). In the same study, we also found that the ability to maintain a neutral pH$_{jm}$ was reduced in rats treated with NH$_4^+$. As the effect of NH$_4^+$ was immediate and thus independent of gene transcription, we concluded that its inhibitory effect on pH$_{jm}$ is non-genomic. Thus, increased expression of SLC26A9 in gastric mucous cells in \textit{H. pylori}-infected mice is likely a
compensatory response to overcome the chronic inhibition of SLC26A9-mediated bicarbonate secretion by \textit{H. pylori}.

In this study the acid-induced hyperemia is absent in \textit{H. pylori}–infected mice. Hence, another important defense mechanism is altered. The finding is somewhat surprising in light of the higher permeability measured in \textit{H. pylori} infected mice, compared to control mice, which would predict an increase in the hyperemic response. One possibility for the blood flow not to respond to acid would be if the resistance vessels already were maximally dilated. This is, however, not probable since the blood flow level in control and infected mice were similar. It is also supported from studies in which blood flow was either not altered or even reduced in chronically \textit{H. pylori} infected mice or human (9, 33, 38). Furthermore, in pilot studies in infected animals we measured an increase in blood flow, when the mucosa was challenged with a stronger acid (pH 0.8).

In a recent study, we showed (29) that the hyperemia induced by luminal acid was absent or substantially reduced in iNOS knock-out mice. Our results also suggested that the iNOS was constitutively expressed in surface epithelial cells. This epithelial NO production might be inhibited by the bacteria residing in the vicinity of these cells. Several suggestions of how \textit{H. pylori} can reduce the formation of NO have been proposed, including the production of an L-arginine analogue, asymmetric dimethyl arginine (ADMA) (12). An arginase produced by the bacteria has also been suggested as a strategy to reduce NO production, as it will consume the substrate for NOS (13). Since the hyperemia seen on challenge with luminal acid in control mice was abolished in the infected mice in our experiments, we may speculate that the bacterium might counteract the acid-induced formation of NO by the epithelial iNOS, and thereby inhibit the signal for hyperemia. However, a hyperemic response was seen on a
higher concentration of acid in mice infected with H. pylori (as described above) which might be due to a direct submucosal acidification and vasodilation.

In conclusion, we have found in this study that a chronic *H. pylori* infection alters the ability of the mucosa to maintain a neutral pH at the epithelial cell surface. This could be due to the thinner inner, firmly adherent mucus gel layer, an increased hydrogen permeability through the mucus and/or to defective bicarbonate transport across the epithelium, e.g., through inhibition of the Cl⁻/HCO₃⁻ exchanger SLC26A9. The latter is most probable involved since we found an increased expression of SLC26A9 mRNA and protein in the superficial mucosa of infected mice. This would have resulted in an increased bicarbonate transport unless the transport was inhibited. In addition, since the hyperemia normally seen in response to luminal HCl is abolished in infected animals, a disturbed signal to the resistance vessels is suggested. Thus, it is apparent that infection with *H. pylori* negatively influences several of the important defence mechanisms in the gastric barrier. It is very likely that the weakened barrier is less resistant to harmful agents but the exact mechanisms leading to development of pathological changes by *H. pylori* remains to be further investigated.
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FIGURE LEGENDS

**Figure 1** Gastric mucosal permeability measured by the clearance of $^{51}$Chromium-labeled EDTA from blood to lumen (ml x min$^{-1}$ x 100 g tissue$^{-1}$) in control mice (n=6) and in mice infected with *H. pylori* (n=4). Values are mean ± SEM. *p<0.05 compared with control animals.

**Figure 2** Mucus thickness and accumulation in control mice (n=5) and in mice infected with *H. pylori* (n=6). A. The values represent the total mucus thickness at the beginning of the experiment and the thickness of the firmly adherent layer after the first removal of the loosely adherent layer. B. The thickness of the mucus layer was measured after removal of the loosely adherent layer and again after 15 and 30 minutes. The loosely adherent layer was then removed again, and the thickness of the firmly adherent layer was measured a second time (second removal). Values are mean ± SEM. *p<0.05 compared with control animals, #p<0.05 compared with results after first removal.

**Figure 3** Juxtamucosal pH in control mice (time 0-25 min: n=7; time 30-45 min: n=5) and mice infected with *H. pylori* 1 (n=5). The loosely adherent mucus layer was removed before the experiment started. *p<0.05 compared with control animals; #p<0.05 compared with baseline levels (time 0-5 min).

**Figure 4** Blood flow measurements in control mice (n=6) and mice infected with *H. pylori* (n=6). Mean arterial blood pressure (MAP) in mmHg, and blood flow (laser-Doppler flowmetry [LDF]) and vascular resistance in percent of the control period at 25-30 min. The mucosa was exposed to HCl (pH 2 and 1.5). The loosely adherent mucus layer was removed
before the experiment started. Values are mean ± SEM. *p<0.05 compared with the control period.

**Figure 5 SLC26A9 expression and scraping of the mucosa**

A. Relative quantification of mRNA from surface epithelial cells from control mice (n=5) and mice infected with *H. pylori* (n=3). The C_T values were used to calculate the amount of PCR product in comparison to G6PDH as a "house-keeping gene", by subtracting the C_T value for G6PDH from the C_T value for SLC26A9 (ΔC_T). Values are mean ± SEM. *p<0.05 compared with control animals.

B. Western blot analysis of SLC26A9 of pooled membrane fractions from control mice (n=3) and mice infected with *H. pylori* (n=3). C. Representative histological sections of unscraped and scraped mucosa. Scale bars are 200 μm.
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