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

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Henriksnäs, Johanna, Mia Phillipson, Martin Storm, Lars Engstrand, Manoocher Soleimani, and Lena Holm. Impaired mucus-bicarbonate barrier in *Helicobacter pylori*-infected mice. *Am J Physiol Gastrointest Liver Physiol* 291: G396–G403, 2006. First published April 13, 2006; doi:10.1152/ajpgi.00017.2006.—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 Lewis b epitopes) and inoculated with *H. pylori*. Mice were anesthetized with isoflurane or Hypnorm-midazolam, the stomach was exteriorized, and the surface of the corpus mucosa was exposed. Mucus thickness was measured with micropipettes, juxtamucosal pH (pHjm) was measured with pH-sensitive microelectrodes, blood flow was measured with laser-Doppler flowmetry, and mRNA levels of the bicarbonate transporter SLC26A9 were 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 mice (31 ± 2 µm) than in control mice (46 ± 5 µm), and no mucus accumulation occurred in infected mice. pHjm decreased significantly more on exposure to luminal acid in infected mice (luminal pH 1.5, pHjm 2.4 ± 0.7) than in control mice (pHjm 6.4 ± 0.5). Despite reduced pHjm, SLC26A9 mRNA expression was significantly, by increased 1.9-fold, in infected mice. The reduction in pHjm 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, e.g., by ammonium, a *H. pylori* product, which has been previously shown to inhibit SLC26A9.

**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 ~50% of the human population (15). A very important factor for *H. pylori* colonization is its cytoplasmic urease activity, which produces 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 pathological changes are still not clear, but disruption of the mucosal barrier is most certainly involved. The only natural hosts 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 uses human α-1,3/4-fucosyl transferase (α-1,3/4FT) to express Lewis b (Leb) epitopes in the surface epithelium was used (10). Leb mediates the 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 preepithelial mucus-HCO$_3^-$ barrier, a tight epithelial barrier, and a subepithelial component including blood flow and nerves. The preepithelial layer involves both secreted mucus and HCO$_3^-$. Next to the epithelial cells, we have identified a firmly adherent mucus layer that is impossible to remove by suction or by wiping with a cotton tip. Luminal 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 (28, 35) have shown that the pH at the epithelial surface [juxtamucosal pH (pHjm)] is neutral despite an acidic lumen. Only the firmly adherent layer is important in maintaining pHjm at a neutral level during luminal acid exposure (28). Thus the firmly adherent layer of mucus together with HCO$_3^-$ 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 the SLC26 family of anion exchangers, has been recently shown to mediate Cl$^-$/HCO$_3^-$ exchange and has been found in the apical membrane of gastric surface epithelial cells (39). In addition to Cl$^-$/HCO$_3^-$ exchange, SLC26A9 can function as a Cl$^-$-independent HCO$_3^-$ transporter. Thus, given its localization and functional modes, SLC26A9 may be important in creating and maintaining the neutrality of pHjm. The SLC26A9-mediated Cl$^-$/HCO$_3^-$ exchange has been shown to be inhibited by NH$_4^+$ (39). This is in contrast with SLC26A3, which is expressed in the colonic epithelium and is actually stimulated by NH$_4^+$ (6). A recent study (39) from our laboratories has demonstrated that the gastric lumen of rats treated with NH$_4^+$ could not maintain neutral pHjm when challenged with acid. 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-HCO$_3^-$ layer by inhibiting SLC26A9-mediated HCO$_3^-$ secretion (39).

Gastric mucosal blood flow has a vital role in gastric mucosal protection. It maintains the juxtamucosal HCO$_3^-$ gradient during acid secretion by transporting HCO$_3^-$ from parietal cells to surface epithelial cells, through which HCO$_3^-$ is secreted into the mucus gel layer. In addition, adequate blood flow to surface epithelial cells is required to maintain the juxtamucosal pH; blood flow; laser-Doppler flowmetry; permeability...
flow is required to maintain the different functions of mucosal cells, including the process of rapid repair. Blood flow is also important in removing backdiffusing hydrogen ions, toxic waste products, accumulated oxygen metabolites, and other inflammatory mediators (17). We have shown in previous studies (29, 35) that luminal acid alone induces hyperemia without any macroscopic lesions. Furthermore, we have suggested that epithelial inducible nitric oxide (NO) 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 gastric mucus thickness and accumulation, pH\textsubscript{inm}, the gastric mucosal blood flow response to acid, and the expression of the Cl\textsuperscript{−}/HCO\textsubscript{3}{−} 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, Sweden, 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\textsuperscript{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). 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 groups; 34 female mice 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; Cockeysville, MD) with 10% horse serum and 8.5% denatured horse blood (chocolate agar) under microaerophilic conditions at 37°C. Bacteria were suspended in sterile PBS solution to a concentration of 10\textsuperscript{7}-10\textsuperscript{8} colony-forming units (cfu)/ml. Organisms were inoculated into the oral cavity with a pipette (50 μl) twice or by gavage (200 μl) four times during a 2-wk period. Control animals were treated with PBS only. Mice were deprived of food and water for 2 h before each inoculation. Animals were between 4 and 12 wk old at the time of inoculation, and the infection was allowed to stabilize for 11–21 wk (average: 15 ± 1 wk).

After the experiment, one-half of the stomach was homogenized in 1 ml of Helicobacter broth (Uppsala University Hospital; Uppsala, Sweden), and 100 μl of the suspension were 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 the presence of urease activity. Animals not confirmed as positive were excluded. In some of the experiments, colonies were counted and expressed as cfu per milligram of 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

Animals were anesthetized with either isoflurane (for mucus measurements, blood flow measurements, and mucosal permeability measurements) or Hypnorm-midazolam (for pH\textsubscript{inm} measurements). Isoflurane anesthesia was induced by spontaneous inhalation (Forene, Abbott Scandinavia; 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 injection of Hypnorm (0.4 ml/kg, giving 0.126 mg/kg fentanyl citrate and 4 mg/kg flumazenil) and midazolam (5 mg/kg) mixed together in a volume of 0.2–0.3 ml. The anesthesia was maintained by a continuous intra-arterial 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 intraperitoneally 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) dissolved in isotonic saline was placed in the carotid artery to monitor blood pressure. The jugular vein was cannulated for the continuous infusion of a maintenance fluid (modified Ringer solution, 0.35 ml/h). In a few experiments, the vein cannula failed, and the infusion was performed via the arterial catheter through a Y connection.

The preparation of the gastric mucosa for intravitral 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 upward. A “mucosal chamber” was fitted over the gastric mucosa, exposing ∼0.13 cm\textsuperscript{2} 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, animals were allowed to stabilize for 20–60 min after surgery.

Mucosal Permeability Measurements

To investigate the effect of H. pylori infection on gastric mucosal integrity, mucosal permeability was determined by measuring the clearance of 51Cr-labeled EDTA (51Cr-EDTA, Perkin-Elmer Life Sciences; Boston, MA) from blood to lumen (26).

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

Mucus Measurements

Micropipettes made of glass tubing [borosilicate tubing, outer diameter (OD) 1.2 mm and inner diameter (ID) 0.6 mm, Haer; Brunswick, ME], pulled to a tip diameter of 1–2 μm and prepared as
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Previously described (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 mucous 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 “digtimatic indicator” (IDC series 543, Mituoyo; 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 total mucous 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 thirty minutes later, mucous thickness was again measured, whereby the rate of mucous 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., pHm, was measured with hydrogen ion-selective microelectrodes as previously described (28). Glass tubing (borosilicate tubing with omega dot, OD 1.2 mm and ID 0.9 mm, Haer) was microelectrodes as previously described (28). Glass tubing (borosilicate tubing with omega dot, OD 1.2 mm and ID 0.9 mm, Haer) 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 HEPS buffer at pH 7.4, which 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. Electrodes were calibrated before and after the experiments in isoosmolar (310 mosM) solutions with a pH of 1.5–8 at 37°C. 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).

Because an earlier study (28) in the rat has revealed that pHm was not influenced by the loosely adherent mucus layer, and because 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, and 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 equipment (Periflux instruments PF3 and PF4001, Perimed; Stockholm, Sweden), which has previously been used to study 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 experiments, the laser light most likely penetrates through the entire thickness of the gastric wall (20). However, the recorded blood flow was mainly mucosal, because the amount of backscattered light decreases exponentially with the depth in the tissue.

The continuously measured blood flow is reported as a percentage of that in the control period, i.e., the 10-min period before the HCl application. After the stabilization period, the loosely adherent mucus was removed, and blood flow was recorded for 30 min, after which the same protocol as for pHm measurements was used.

SLC26A9 Expression

Surface epithelial cells. Two-thirds of the stomach from H. pylori-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 one-third 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 a surface epithelial cell preparation of five control and three H. pylori-infected animals. cDNA was synthesized using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) with random hexamers according to the protocol from the manufacturer.

A Rotor-Gene 3000 (Corbette Research) and detection with SYBR green were used to amplify and analyze generated cDNA. The primers used were glucose-6-phosphate dehydrogenase (G6PDH; Genebank Accession No. BC075663), 5’-ATTGACCATCCTGGGCAA-3’ and 5’-CCCTGATGATCCCAAATCTCA-3’, and SLC26A9 (Genebank Accession No. AK086815), 5’-TGTTCTTCTCCAGGCTT-GCC-3’ and 5’-CCAGTGTGAGCCATTTCA-3’.

PCR amplifications were performed in a total volume of 25.5 μl, containing 8 μl cDNA, 0.3 μM 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 for 20 s, 59°C for 30 s, and 72°C for 40 s. The results are presented as threshold cycle (Ct) values, i.e., the estimated amplification cycle number when the fluorescence exceeds a specified threshold value. All samples were run in duplicate. Ct values were used to calculate the amount of PCR product compared with G6PDH as a “housekeeping gene” by subtracting the Ct value for G6PDH from the Ct value for SLC26A9 (ΔCt).

Semiquantitative immunoblot analysis. A total cellular fraction containing plasma membrane and intracellular membrane vesicles was prepared from pooled gastric surface epithelial cell preparations from three control and three H. pylori-infected animals. Tissue samples were homogenized in ice-cold isolation solution (250 mM sucrose and 10 mM triethanolamine, pH 7.6) containing protease inhibitors (0.1 mg/ml phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin) using a Polytron System PT 1200 CL (Kinematica). The homogenate was centrifuged at low speed (1,000 g) for 10 min at 4°C to remove nuclei and cell debris. The supernatant was centrifuged at 150,000 g for 90 min at 4°C. The pellet containing plasma membrane and intracellular vesicles was suspended in isolation solution with protease inhibitors.

Membrane fractions were solubilized at 65°C for 20 min in 5X Laemmli buffer (0.125 M Tris, 0.86 M glycerine, and 1% SDS), and the total protein concentration was measured with a DC protein assay (Bio-Rad; Hercules, CA). One hundred micromicrograms of total protein per well were loaded onto 10% polyacrylamide minigels (Ready Gel Precast Gels, Bio-Rad), size fractionated under denaturing conditions, and then transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with 8% milk proteins and then probed with afffinity-purified anti-SLC26A9 immune serum. Goat anti-rabbit IgG antibody coupled to horseradish peroxidase (Bio-Rad) was used as a secondary antibody. Proteins were visualized by enhanced chemiluminescence detection (ECL+; Amersham Biosciences). SLC26A9-positive bands were quantified with Quantity One software (Bio-Rad).

Chemicals

The chemicals used were as follows: heparin (Leo Pharma), Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone, Janssen Pharmaceutica; Beerse, Belgium), midazolam (5 mg/ml Dormicum, Roche; Stockholm, Sweden), silicone grease (Dow Corning high vacuum grease, Dow Corning; Weisbaden, Germany), HEPES (Merck; Darmstadt, Germany), tributylchlo罗斯iliane (Fluka), and bovine albumin (Sigma-Aldrich Chemie; Steinheim, Germany). DNA was purified and amplified using a DNeasy Tissue Kit and Master Mix

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No ulcers were detected, but small mucosal bleeding was observed in one H. pylori-infected stomach compared with uninfected controls. 

\[ H. \text{pylori} \times 3.8/1000 \text{cells} \times 10^6 \text{cells} \]

mice (n = 6) and in mice infected with Helicobacter pylori (n = 4). Values are means ± SE. *P < 0.05 compared with control animals.

(Qiagen; Hilden, Germany). The modified Ringer solution contained 120 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, and 0.75 mM CaCl₂.

Statistics

All values are presented as means ± SE. 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; Berkeley, CA) were used for all statistical calculations.

RESULTS

Infection

No H. pylori was found in the noninoculated 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 the groups (control mice 135 ± 8, n = 8, and 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 compared with uninfected controls. No ulcers were detected, but 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 (Fig. 1). We did not observe any gender differences in any of the parameters investigated.

Mucus Measurements

Results from the mucus measurements are presented in Fig. 2, A and B. The total mucus thickness (both loosely and firmly adherent) did not differ between the controls (n = 5) and infected (n = 6) animals. However, when the loosely adherent layer was removed by gentle suction, a significantly thinner firmly adherent layer was revealed in infected animals. Thirty minutes after the first mucus removal, the mucus thickness had increased significantly in control animals, and the mucus accumulation was calculated to be 9 ± 3 μm or 0.3 ± 0.1 μm/min. In infected animals, no significant mucus accumulation had occurred (3 ± 1 μm or 0.1 ± 0.04 μm/min). 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 pHₐm in control (n = 7) and infected (n = 5) animals. pHₐm was neutral during the control period in both groups. When HCl (pH 2 and 1.5) was applied luminally in the control animals, pHₐm decreased significantly to 6.6 ± 0.4 (transiently) and 6.3 ± 0.6, respectively. Infected animals, pHₐm 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), pHₐm decreased continually to 2.4 ± 0.7 and did not return to the control level until 10 min after removal of the acid. There was a significantly greater decline in pHₐm in infected animals than in controls during luminal application of HCl at both 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 shows the 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 was 301 ± 92 perfusion units/g stomach in controls and 292 ± 43 perfusion units/g stomach in infected animals.

SLC26A9 Expression

The procedure of scraping the mucosa removed only the uppermost part of the mucosa, as revealed histologically (Fig. 5C). The expression of SLC26A9 mRNA in surface epithelial cells from H. pylori-infected mice increased significantly (by 1.9-fold) when measured by real-time PCR. The ΔCT values are shown in Fig. 5A (control, n = 5, and infected, n = 3). The results of semiquantitative Western blot analysis of SLC26A9 showed the upregulation of the expected ~80-kDa band by approximately fourfold in H. pylori-infected animals (Fig. 5B; control, n = 3, and infected, n = 3).

DISCUSSION

The mucus-HCO₃⁻ 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.

In the present study, we used a special mouse model, α-1,3/4FT-expressing mice, infected with a human H. pylori strain (Hp1). α-1,3/4FT mice use human α-1,3/4FT to express Leb epitopes in their pit region and surface mucus cells (14). Leb is a blood group antigen that is recognized by an H. pylori adhesin, blood group antigen-binding adhesin (BabA) (19). Thus the presence of Leb in the host and BabA in the bacteria mediates adherence to the gastric epithelial lining and implies a supposedly more severe infection. Indeed, the α-1,3/4FT mice developed 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 (28) have also concluded that it is the firmly adherent layer that is important for the protection against luminal acid, because it is in this layer that the pH gradient is formed. 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 infected mice was significantly thinner than in noninfected controls, but was still a continuous layer. Furthermore, no mucus accumulation was detected during the 30-min measuring period. Clearly, mucus does accumulate also in infected animals, because we found the same total mucus thickness in

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

**Fig. 4.** Blood flow measurements in control mice (n = 6) and mice infected with H. pylori (n = 6). Top: mean arterial blood pressure (MAP; in mmHg). Middle: blood flow [by laser-Doppler flowmetry (LDF)]. Bottom: vascular resistance (R; as a percentage of the control period at 25–30 min). The mucus was exposed to HCl (pH 2 and 1.5). The loosely adherent mucus layer was removed before the experiment began. Values are means ± SE. *P < 0.05 compared with the control period.
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), MUC secretion reduced (36), or mucins degraded (24). Furthermore, the expression patterns of gastric mucins MUC5AC and MUC6 seem to be altered in _H. pylori_-infected patients and skewed toward more MUC6 (5, 37). Measurements 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 the accumulation rate. We found that the total mucus thickness (loosely + firmly adherent mucus) did not differ between infected and noninfected 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 HCO₃⁻ neutralizes backdiffused acid and a pH gradient is formed, in pH advanced to mucosal cells (30, 32). This was also shown to occur in control mice in the present study. However, the infected mice could not preserve neutral pHₘₚ in the presence of luminal acid. _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 the maintenance of the pH gradient. An in vitro study (7) has suggested that the presence of NH₄⁺, which is produced by the _H. pylori_ urea-splitting enzyme urease, could increase the permeability of protons in the mucus.

Another possibility is that _H. pylori_ inhibits HCO₃⁻ transport across epithelial cells or the expression of HCO₃⁻ transporters. In this study, however, we observed increased mRNA expression and protein abundance of an important Cl⁻/HCO₃⁻ exchanger, SLC26A9, in gastric surface epithelial cells in _H. pylori_-infected mice. On the basis of this observation alone, an obvious conclusion would have been that HCO₃⁻ transport would increase. However, because the in vivo measurements showed reduced pH at the surface of epithelial cells, an inhibition of HCO₃⁻ transport most probably occurred. Interestingly, we have shown in an in vitro study (39) that SLC26A9 is inhibited by the _H. pylori_ product NH₄⁺. In the same study, we also found that the ability to maintain neutral pHₘₚ was reduced in rats treated with NH₄Cl. Because the effect of NH₄⁺ was immediate and thus independent of gene transcription, we concluded that its inhibitory effect on pHₘₚ was nongenomic. Thus increased expression of SLC26A9 in gastric mucous cells in _H. pylori_-infected mice is likely a compensatory response to overcome the chronic inhibition of SLC26A9-mediated HCO₃⁻ secretion by _H. pylori_.

In this study, acid-induced hyperemia is absent in _H. pylori_-infected mice. Hence, another important defense mechanism is altered. This finding is somewhat surprising in light of the higher permeability measured in _H. pylori_-infected mice compared with 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 were already maximally dilated. This is, however, not probable because the blood flow levels in control and infected mice were similar. It is also supported by results from studies (9, 33, 38) in which blood flow was either not altered or even reduced in chronically _H. pylori_-infected mice or humans. 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 (29) showed that the hyperemia induced by luminal acid was absent or substantially reduced in iNOS knockout mice. Our results also suggested that 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 _H. pylori_ can reduce the formation of NO have been proposed, including the production of an l-arginine analog.
asymmetric dimethyl arginine (12). An arginase produced by the bacteria has also been suggested as a strategy to reduce NO production, because it will consume the substrate for NOS (13). Because the hyperemia seen on challenge with luminal acid in control mice was abolished in infected mice in our experiments, we may speculate that the bacterium might counteract the acid-induced formation of NO by 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 direct submucosal acidification and vasodilatation.

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 defective HCO\(_3\)_\(^-\) transport across the epithelium, e.g., through inhibition of the Cl\(^-\)/HCO\(_3\)_\(^-\) exchanger SLC26A9. The latter is most probably involved because we found increased expression of SLC26A9 mRNA and protein in the superficial mucosa of infected mice. This would have resulted in increased HCO\(_3\)_\(^-\) transport unless the transport was inhibited. In addition, because the hyperemia normally seen in response to luminal HCl was abolished in infected animals, a disturbed signal to the resistance vessels is also 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 the development of pathological changes by _H. pylori_ remain to be further investigated.

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