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Gastroduodenal Mucus Gel Thickness in Patients with *Helicobacter pylori*: A Method for Assessment of Biopsy Specimens

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The gastroduodenal mucus layer is considered the primary mucosal protective barrier, especially important in the maintenance of a mucosal pH gradient. Thus, the measurement of the mucus layer thickness in various disease states could advance our understanding of gastroduodenal pathophysiology. We present a novel method for measuring the mucus layer in endoscopic biopsy material and compare layer thickness in *Helicobacter pylori* (HP)-negative and HP-positive specimens. Endoscopic biopsies were obtained from 17 patients with gastroduodenal mucosa harboring HP and from 15 patients without current HP colonization. The thickness of the mucus layer was measured in fresh specimens by the phase-contrast dark-field microscopy technique. In patients with confirmed HP infection, the thickness of the mucus layer (mean ± SD) was 0.093 ± 0.033 mm in duodenal, 0.085 ± 0.027 mm in antral, and 0.105 ± 0.033 mm in corporal mucosa. In patients without concomitant HP colonization, the thickness of the mucus gel was 0.162 ± 0.045 mm, 0.175 ± 0.067 mm, and 0.161 ± 0.064 mm in duodenum, antrum, and corpus, respectively. The differences between the means were statistically significant ($p < 0.001$ for the duodenal, $p < 0.001$ for antral, and $p < 0.01$ for corporal mucosa). This study suggests that colonization of the gastroduodenal mucosa by HP impairs the mucus layer covering the surface epithelium. This mucus layer impairment may lead to mucosal injury with subsequent development of inflammation and, possibly, peptic ulcer disease.

INTRODUCTION

Mucosal protection strongly depends upon the dynamic equilibrium between damaging and protective factors operating within the alimentary tract mucosa. Since most known damaging factors, both exogenous and endogenous, act on the luminal side of the mucosal compartment, the mucus layer plays a primary role in mucosal defense (1–5).

Helicobacter pylori (HP) resides within the mucus layer and could alter the structure and function of the gastroduodenal mucus. Numerous *in vitro* studies have demonstrated that HP generates many enzymes that can influence the biology of surface epithelium. These enzymes indirectly affect the mucus layer by changing the synthetic and secretory activities of mucous cells (6). Some of these enzymes, including protease, lipase, and phospholipase A2, may also directly affect the function of the mucus layer through proteolysis of mucus glycoprotein and lipolysis of mucus-associated lipids, especially phospholipids, which are crucial for inhibition of hydrogen ion back-diffusion (7–10).

Although the effect of HP enzymes on the physicochemical properties of gastric mucus has been well studied *in vitro* (7–10), these results have not been confirmed *in vivo* due to the lack of appropriate methodology. We report a newly developed method designed to measure the thickness of the mucus layer in endoscopically obtained biopsy specimens. We then utilized this method to relate mucus layer thickness to the presence or absence of HP.

MATERIALS AND METHODS

The study population consisted of 32 patients consecutively presenting for a routine diagnostic endoscopy at the Gastroenterology Clinic, University of Virginia Health Sciences Center. The study was approved by the Human Investigation Committee, and informed consent was obtained from all study patients. Patients fasted for up to 12 h before endoscopy.

Biopsy specimens were taken from three locations: *1*) 3 cm beyond the pylorus on the anterior wall of the duodenal bulb, *2*) on the greater curve of the antrum 5 cm proximal to the pylorus, and *3*) on the greater curve of the body mucosa in an area with prominent gastric folds. Gastric and duodenal biopsies were obtained at least 1 cm from any mucosal lesion.

Immediately after biopsy, and before HP status was

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known, biopsy samples for mucus thickness estimation were transported to the laboratory for independent evaluation.

The diagnosis of HP was confirmed by histology, culture, CLOtest, ELISA, and [^{14}C]urea breath test.

The biopsies for light microscopy were fixed in 10% formalin, routinely processed to paraffin, and 3-μm sections were cut. Sections were stained with hematoxylin and eosin, and by the Warthin-Starry technique.

Culture of HP was performed on biopsy specimens homogenized and inoculated into horse blood agar plates with GCHI enrichment (Remel). Plates with inoculated samples were subsequently kept in a 10% CO_2 incubator for 7 days.

CLOtest was performed with standard plates supplied by Tri-Med Specialities Inc.

ELISA was conducted on microtiter plates covered with HP antigen extracted by 0.2 M glycine hydrochloride; a second antibody, phosphatase-labeled goat anti-human IgG antiserum (Kpl), was also used.

The [^{14}C]urea breath test was performed on fasting patients. Patients were advised to drink a solution of 5 μCi of [^{14}C]urea and to blow expirated air through a solution entrapping traced carbon dioxide at 2, 4, 8, 12, and 20 min. Samples of solutions containing tracer were counted with a β-scintillation counter.

The thickness of the mucus layer was measured according to the method of Kerss *et al.* (11), but modified as we have previously described (12). This method has been currently adapted to conditions required for processing of human biopsy samples obtained during endoscopy.

The biopsy specimen, luminal surface up, was placed in sterile 0.15 M PBS pH 7.4 on a Millipore filter. Sections of mucosa, separated by a distance of 0.7 mm, were cut with a sharp triple razor blade. Two parallel slices of mucosa were obtained and placed transversely on a sterile Petri dish so that they could be exposed to side-view illumination under an inverted microscope. Positioning of sections was checked stereoscopically (×9 magnification) to insure that they were not mounted obliquely. Parallel samples, remaining in the sterile Petri dish, were placed in a 5% CO_2 incubator for 15 min to allow full restoration of the mucus layer from the strain applied during the sampling procedure. Throughout the entire procedure, extreme care was taken to maintain conditions as close as possible to that *in vivo*.

The thickness of the mucus layer was measured by means of an inverted microscope (Carl Zeiss, Germany) with dark field illumination (×78.75 magnification). The distance between the luminal edge of the mucus layer and mucus-epithelium interface was measured by an eye-piece graticule standardized with a stage micrometer. Mean thickness of the mucus layer of each duplicate specimen was computed from a minimum of 12 measurements (six recordings for each specimen at intervals of 0.250 μm along the luminal edge of specimen).

After the mucus thickness data had been recorded independently on 32 patients, they were subsequently divided into two subgroups with respect to their HP colonization status: HP-positive and HP-negative.

In a separate experiment, fresh piglet stomachs (Archer Farms, MD) were opened along the greater curvature, and samples of the gastric mucosa were prepared, as described above, using both the whole thickness of the gastric wall and fragments obtained with biopsy forceps. This experiment was performed to study the sample-processing error resulting from the biopsy procedure-related stress.

Results are presented as means ± SD, and statistical analysis was evaluated by Student's unpaired t test performed with the StatSoft software package.

RESULTS

The HP-positive group comprised 17 patients (10 male and seven female; average age 49 yr, range 30–79). The HP-negative group consisted of 15 patients (nine male and six female; average age 51 yr, range 34–79). Two duodenal ulcer patients, one gastric ulcer patient, and 14 patients with nonulcer dyspepsia comprised the HP+ group. The HP− group consisted of one patient with duodenal ulcer, one patient with gastric ulcer, and 13 patients with nonulcer dyspepsia.

The HP− population was not statistically different from the HP+ group with respect to age, sex, or underlying gastrointestinal disease.

Dark field illumination generated a bright picture of the mucus layer which was optically distinct from both the luminal and mucosal compartments with a clear contrast delineating their borders (Fig. 1, A and B). Figure 1A represents a fragment of the thinnest mucus layer from all studied specimens, whereas Figure 1B illustrates a more representative appearance of the studied mucosa. The thickness of the mucus layer varied between patients, and even within individual specimens, with some areas exhibiting a mucus layer approximately 2–4 times thicker than others. The mean values and ±SD of mucus gel thickness sampled from the three sites in studied subgroups are presented in Table 1.

In HP− patients, there was no significant difference in mucus gel thickness measured in duodenum, antrum, and body of the stomach. However, there was a tendency toward a thicker mucus layer in the antral mucosa. In HP+ patients, the thickness of the mucus gel was decreased by 52% in antrum, 43% in duodenum, and 35% in the body of the stomach, indicating that the thickness of the antral mucosa was most pro-

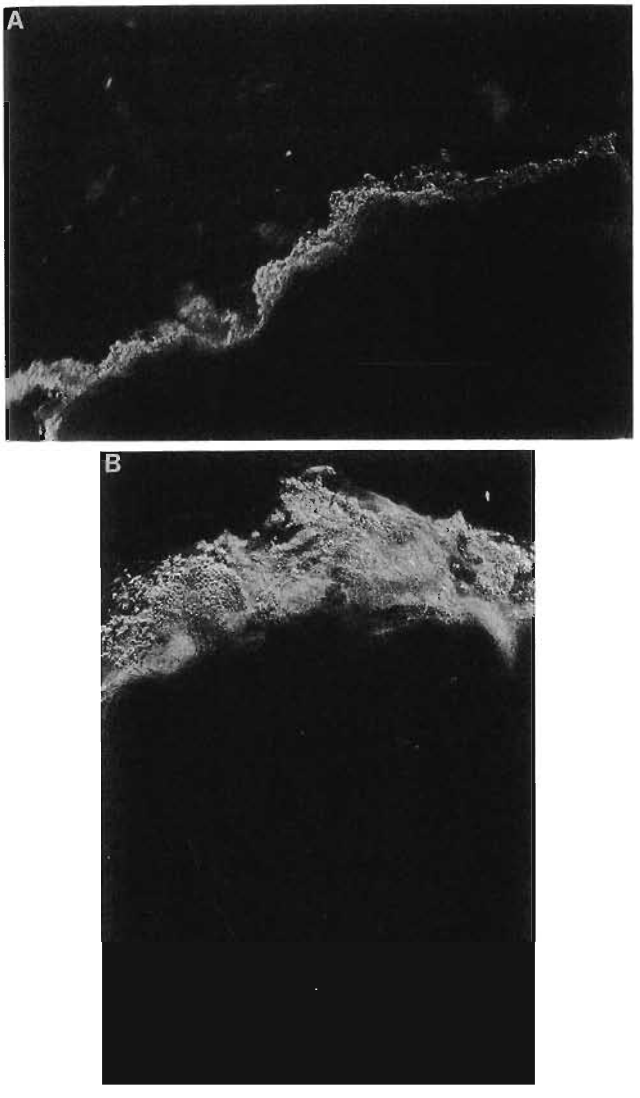

FIG. 1. The thickness of the mucus layer in a specimen of antral mucosa of patients with HP-related gastric pathology visualized by dark-field microscopy techniques (×78.75). The bright color represents a mucus layer visualized between the dark field of bathing solution on the luminal side of the mucosa and the dark background of the underlaying epithelium. *A*, the minimal mucus layer preserved on the surface epithelium; *B*, the more representative thickness of the mucus layer in all studied specimens.

TABLE 1

Thickness of the Mucus Layer in Human Corporal, Antral, and Duodenal Mucosa Colonized or Noncolonized by Helicobacter pylori

Group Studied	Thickness of Mucus Layer (mm)		
	Corpus	Antrum	Duodenum
HP-positive	0.105 ± 0.033	0.085 ± 0.027	0.093 ± 0.033
HP-negative	0.161 ± 0.064	0.175 ± 0.067	0.162 ± 0.045
t	−2.965	−5.332	−4.865
$p <$	0.01	0.001	0.001

foundly affected by colonization with the microorganism.

The difference between the mean thickness of the mucus layer for HP-positive patients as compared to HP-negative patients was statistically significant ($p < 0.001$ for duodenal, $p < 0.001$ for antral, and $p < 0.01$ for corporal mucosa) (Fig. 2).

The thickness of the pig gastric mucus gel measured in specimens obtained through resection of the gastric wall and from the parallel biopsy procedure were 0.276 ± SD 0.091 mm and 0.263 ± SD 0.098 mm, respectively.

This parallel measurement of the thickness of the gastric mucus layer using well-established conventional dissection method and our newly developed biopsy procedure served well to validate the accuracy of our methodology.

DISCUSSION

The integrity of the mucus layer on the surface of the epithelium is dependent upon the viscoelastic properties of the major mucus component (mucus glycoprotein), so-called mucin. The viscosity of mucus glycoprotein polymer and its ability to adhere to the cell membrane structures of the surface epithelium is significantly enhanced through its interaction with mucus-associated lipids, especially phospholipids, albumin, and immunoglobulins (2, 3). Moreover bicarbonate, generated by the surface epithelium, is trapped in the architectural network of the mucus glycoprotein polymer within the unstirred mucus layer. This mucus-bicarbonate interface helps to maintain a pH gradient, preserving neutral pH at the surface of the epithelial cells despite the highly acidic luminal milieu (4, 5).

The luminal side of the mucus layer undergoes continuous erosion due to the activity of pepsin, the major protease of the gastric juice. The rate of proteolysis is strongly enhanced by the low pH of gastric milieu, whereas the presence of sulfated glycoprotein, mucus-associated lipids, especially sulfated glycolipids, and pH of gastric secretion shifting toward neutral or alkaline are all factors promoting resistance to proteolysis of the gastric mucus layer (18).

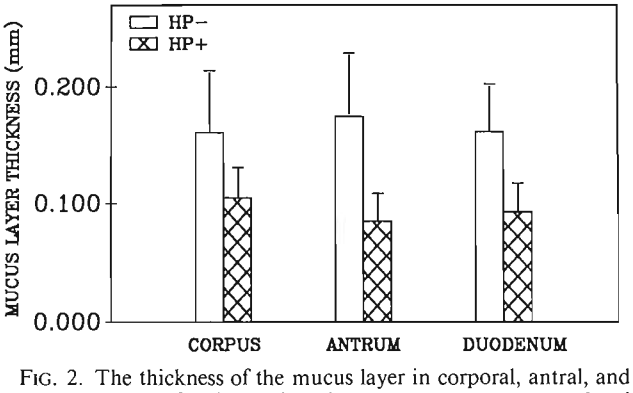

FIG. 2. The thickness of the mucus layer in corporal, antral, and duodenal mucosa of patients with alimentary tract symptoms related to HP colonization (HP+), and unrelated to HP infection (HP−). The difference was statistically significant ($p < 0.001$ for the duodenal, $p < 0.001$ for antral, and $p < 0.01$ for corporal mucosa).

Thus, the thickness of the mucus layer is maintained through an equilibrium between the rate of biosynthesis and secretion of mucus from the mucous epithelial cells including the degree of subsequent polymerization on the surface of mucosa, the extent of hydrophobic interaction with lipids and proteins, degree of absorption of continuously secreted bicarbonate), and the rate of proteolytic degradation on its luminal interface (1, 2).

This dynamic balance within the mucus layer between degradation and secretion may explain, at least in part, why Wallace (23), who recently considered a mucus-bicarbonate barrier as functionally redundant, did not see a potentiating effect of mucolytic agents on mucosal damage caused by hydrochloric acid in this otherwise very elegant paper. Even if this group had found an increase of Alcian blue-binding activity in the bathing solution after mucolytic agents, the thickness of the mucus layer could have been totally restored due to secretion stimulated by back-diffusing hydrogen ions. Only measurement of the mucus thickness after mucolytic agent activity could have properly addressed their findings. Moreover, any electrode introduced to the mucosal surface through the mucus layer automatically compromises the mucus barrier as a result of an artificial channel created around the electrode facilitating flux of hydrogen ions into the mucosa. This may explain why the pH of the surface of the mucosa, reported by the same author, was merely 5, even at the beginning of the experimental procedure, when the pH of bathing solution was 2.0. Only an electrode introduced into the mucus-mucosal epithelium interface from the serosal side could properly approach the measurement of the pH gradient while the bathing solution contains a gradient of hydrogen ion from 0.1 to 1 million on the luminal side of the mucosa. We strongly believe that it is important that this type of study be performed in the near future, to clarify the controversy surrounding both the pH gradient and the role of the mucus-bicarbonate barrier (23–25).

Thus, the measurement of the mucus layer thickness, both *in vitro* and *in vivo*, may provide important insight into the ultimate functional balance or disequilibrium between a variety of aggressive and protective factors acting within an unstirred layer of this primary mucosal defense.

The thickness of the mucus layer can be measured *in vitro* by a slit lamp and pachymeter technique (19), originally used in ophthalmology for evaluation of the cornea, by dark-field microscopy (11), or by direct light microscopy (20). The slit lamp and pachymeter technique require a large fragment of mucosa, available only during surgery, and implementation of a correction factor when the thickness of the measured layer exceeds 0.5 mm, as a nonlinear error may reach 10% (21). Finally, it may also be difficult to differentiate optically the refractive index of extracellular mucus attached to the surface epithelium from intracellular mucin under the apical cell membrane of surface epithelium.

These technical difficulties may at least in part explain why the thickness of the gastric mucus layer measured by slit lamp and pachymeter technique is more than twice the thickness recorded by the dark-field microscopy method. This later technique, while simple and reliable for assessment of the surface mucus gel, to date in human studies has been applied only to large, surgically resected specimens. We have adapted this methodology for the measurement of the mucus layer in pinch biopsy specimens obtained during routine diagnostic endoscopy.

To study the impact of the pinch biopsy procedure on gastric tissue, to validate and to establish the reliability of our modified methodology, we simultaneously studied fresh pig stomach fragments obtained both by surgical resection and endoscopic forceps. The difference in the recorded thickness was 4.7% and well within acceptable limits of methodological error.

The role of HP in the pathology of both gastric and duodenal mucosa has been clearly established, although the exact mechanism of the damage to the colonized epithelium still remains elusive (13–16). As has been

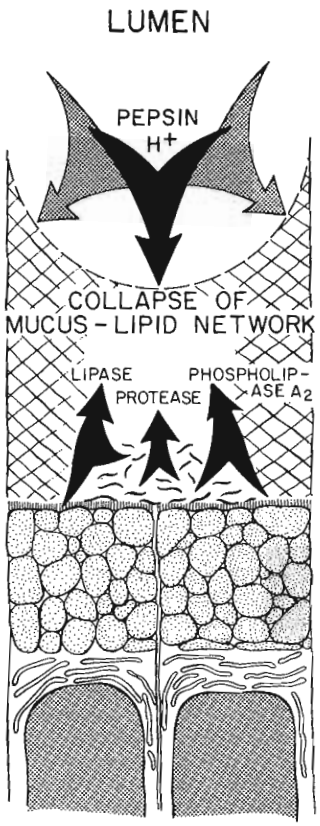

FIG. 3. Schematic presentation of the possible mechanism of damage to the mucus gel layer by HP colonizing the surface epithelium. ~, HP bacterium.

recently shown, the HP microorganism is able to elaborate protease, lipase, and phospholipase A2, all capable of degrading the major components of gastric mucus; *i.e.*, mucus glycoprotein and mucus-associated lipids, especially phospholipids. This degradation may compromise the mucus-bicarbonate barrier, and facilitate the back-diffusion of hydrogen ions with all the pathophysiological consequences (8–10, 17).

The results of our human study clearly indicate that, in patients whose gastroduodenal mucosa harbors HP, the thickness of the mucus layer is significantly diminished, compared with an age- and gender-matched group of patients free of this microorganism. HP affected the mucus layer in both the stomach and duodenum. However, within the stomach, the antral mucus layer was more affected than corporal mucus. This may partially explain the tendency for the antral location of HP-related gastric pathology.

The impairment of the mucus layer by HP may result in pathophysiological consequences (Fig. 3). The decrease of mucus layer thickness could be explained by protease and phospholipase A2 elaborated by *Helicobacter pylori* (8–10). Protease might lead to degradation of the architectural glycoprotein framework supporting the structural unit necessary for the function of the mucus gel. Phospholipase A2 might directly degrade phospholipids, crucial for hydrophobicity of gastroduodenal mucus, or generate lysolecithin (lysophosphatidylcholine) which, in turn, exerts detergent-like activity against both mucosal epithelial cell membranes and lipids imbedded in the glycoprotein polymer. These changes would lead to a collapse of the mucus layer, especially in the vicinity of the surface epithelium. Adhesion of the mucus layer to the mucus cell membranes would then be inevitably lost.

Changes in the physicochemical properties of the mucus layer would allow increased permeability to hydrogen ions and augment susceptibility to proteolytic degradation by pepsin, further facilitating erosion on its luminal surface and subsequently abolishing any functional pH gradient.

Although the above pathogenic scenario is attractive, one cannot completely exclude the possibility that the mucus thickness is in some way primarily compromised, which, in turn, facilitates colonization by HP. However, intrafamiliar clustering of HP, despite obvious differences in genetic constitution between both spouses, casts doubts as to the role of preinfective status of the mucus layer in HP colonization (26). Only a detailed study of the mucus thickness before and after HP eradication would totally exclude the hypothetical role of other factors, such as environmental, which could also potentially lead to the decrease of the mucus layer thickness, thus facilitating mucosal HP inoculation.

The HP microorganism possesses an enormous urealytic activity, able to neutralize diffusing hydrogen ions through generation of ammonia and bicarbonate. However, urease has a cytopathic effect on the epithelium (22)—therefore, it may directly impair the synthesis and secretion of bicarbonate and mucin. In the case of profound changes in the mucus layer with concomitant exposure to a highly acidic gastric milieu, the microorganism may have to restrain its biological activity or move closer to the surface of epithelium coming in intimate contact with epithelial cells. It may, in turn, increase exposure of epithelium to HP cytotoxins, and initiate the cascade of arachidonic acid metabolism, with subsequent elevation of chemotactic factor, accumulation of granulocytes, and the development of active inflammation. In addition, in order to survive, HP may migrate toward the healthy epithelium in a surrounding area that has still well-preserved ability to synthesize and secrete the major components of the mucus layer. The area with both an impaired mucus layer due to colonization with HP and a metabolically weakened surface epithelium may become an easy target for a highly acidic gastric milieu, with the subsequent development of erosions and/or ulcers.

Our method of measurement of mucus layer thickness seems to be a new and reliable tool for the assessment of the protective quality of gastroduodenal mucosa in health and disease. It may serve not only for the study of pathophysiology of the upper alimentary tract mucosa, but also for clinical evaluation of the efficacy of antiulcer drugs developed to enhance mucosal protective mechanisms.

This study suggests that colonization of the gastroduodenal mucosa by HP impairs the mucus layer covering the surface epithelium, which in turn may lead to mucosal injury and subsequent development of inflammation and peptic ulcer disease.

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