Inflammatory Responses and Intensity of *Helicobacter pylori* Infection in Patients with Duodenal and Gastric Ulcer: Histopathologic Analysis with a New Stain

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**Background:** The basic histopathologic patterns of *Helicobacter pylori* gastritis have been described, but the details of the interaction between bacteria, epithelial cells and inflammatory cells are poorly understood. One of the limiting factors is the lack of a staining technique allowing the simultaneous visualization of bacteria and the morphologic details of the infected mucosa.

**Purpose:** To use a new stain to analyze intensity and distribution of *H. pylori* and gastritis in patients with *H. pylori* gastritis without ulcer, with duodenal ulcer, and with gastric ulcer, in an attempt to elucidate the role of *H. pylori* in the pathogenesis of peptic ulcer disease.

**Methods:** patients underwent esophago-gastro-duodenoscopy and gastric biopsies were obtained from 3 sites on the antral lesser curvature, 2 on the antral greater curvature, and 6 on the corpus. Formalin-fixed biopsy specimens were processed, oriented, embedded in paraaffin, cut in 4-µm sections and stained with a combined stain developed in our laboratory (a combination of hematoxylin and eosin with a silver stain and Alcian Blue at pH 2.5). This stain allows the simultaneous visualization of *H. pylori*, the gastric mucosal morphology, and the inflammatory infiltrate. Density of *H. pylori*, neutrophils and mononuclear cells were scored on each specimen using a scale from 0 to 5.

**Results:** Density and distribution of *H. pylori* were essentially similar in the non-ulcer infected patients and in patients with gastric ulcer. Patients with duodenal ulcer had a higher density of bacteria in the antrum than in the corpus. All patients had significantly greater inflammatory responses in the antrum than in the corpus, but this gradient was much more pronounced in patients with duodenal ulcer than in those with gastric ulcer and those with no ulcer. Surprisingly, the strongest intensity of gastritis (both in the antrum and corpus) was found in this latter group.

**Conclusions:** The gastritis gradient is more important than the intensity of inflammatory responses per se in determining the outcome of *H. pylori* infection with respect to the development of peptic ulcer. Larger studies, using new methodologies that allow a better assessment of the numbers of bacteria in the gastric mucus, should be performed to better elucidate the relationship between magnitude of infection, intensity of the mucosal inflammatory responses, and outcome of *H. pylori* gastritis.

Key words: Gastritis, Helicobacter pylori, Ulcer, Peptic ulcer, Special stains, Histopathology

I. Introduction

*Helicobacter pylori*-associated chronic active gastritis is probably the most prevalent chronic infection in the world. Its association with peptic ulcer disease is undisputed [4, 6, 13], and possible connections with gastric carcinoma and primary gastric lymphoma are becoming increasingly apparent [1, 19]. Although the basic histopathologic patterns associated with *H. pylori* infection have been described [2, 8–10], the relative importance of the distribution of *H. pylori* and the intensity of the
associated inflammatory responses in different areas of the stomach with respect to the risk for either peptic ulceration has rarely been addressed [21].

An important limiting factor in the histopathologic study of *H. pylori*-gastritis has been that the lack of a staining technique that would permit to simultaneously observe the bacteria and the morphologic details of the mucosa. This has so far prevented an accurate assessment of the details of the interaction between bacteria, epithelial cells and inflammatory cells. Bacteria lying within the mucus and on the epithelial surface can usually be seen on sections stained with hematoxylin and eosin (H&E), but those closely adherent to cells or located between cellular spaces are frequently undetected.

Silver-based stains (Warthin-Starry and Steiner's) and variously modified Romanowsky-type stains (Giemsa and Diff-3) and Gram stains demonstrate bacteria efficiently and are commonly used in histopathology laboratories [16, 17, 24]. Other stains (toluidine O, acridine-orange) have occasionally been proposed, but have not received wide acceptance [13, 23]. Immunohistochemical methods are highly specific and have an important role in selected situations [14], but they are expensive and labor-intensive; therefore, they cannot be advocated for the routine diagnosis of *H. pylori*-gastritis. All these techniques, however, fail to reveal the tissue morphology in a fashion adequate for evaluation. Thus, investigators have assessed the bacteriologic and morphologic aspects of the infection by using different stains on different slides. One problem with this approach is that the section on which bacteria are quantitated is not the same on which the inflammatory responses are evaluated.

We have recently developed a novel staining procedure that allows to observe the histopathologic characteristics of the tissue while optimally demonstrating *H. pylori* [11]. By using this new technique in combination with a systematic bioptic protocol of the gastric mucosa (gastric mapping) developed in our unit [7], we have tested the hypothesis that different distribution of the bacteria and different intensity of the gastritis account for the different expression of *H. pylori* infection.

II. Materials and Methods

**Study subjects**

This study was approved by the Human Research Committee of Baylor College of Medicine. Informed consent was obtained from 84 subjects (68 men and 18 women, age 23–74 years). Twenty-four subjects were volunteers with no evidence of gastrointestinal disease, negative urea breath test [12] and no serum antibodies against *H. pylori* antigens [3]: this group is referred to as the "normal uninfected subjects." Nineteen subjects had *H. pylori* infection and no evidence of ulcer disease; 21 patients had *H. pylori* infection and gastric ulcer; 20 patients had *H. pylori* infection and duodenal ulcer. The occasional use of non-steroidal anti-inflammatory drugs could not be excluded with certainty in all subjects; however, none of them was a current user of these medication.

**Endoscopy and sampling**

All subjects underwent esophago-gastro-duodenoscopy and gastric biopsies were obtained using a large cup forceps with the spike removed (13K, Olympus, Lake Success, NY) according to our previously described complete mapping protocol [7]. Briefly, biopsies were obtained from the following predetermined sites: A1 (antral lesser curvature, within 2 cm. from the pylorus), A2 (2 cm. proximal to A1); A3 (lesser curvature, incisura angularis); A4 (greater curvature, within 2 cm. from the pylorus); A5 (2 cm. proximal to A4); B1 (lesser curvature, 2 cm. proximal to the angulus); B2 (2 cm. proximal to B1); B3 (greater curvature, where the folds begin); B4 (4 cm. proximal to B3); B5 (middle portion of corpus, greater curvature); B6 (traditional anatomic fundus, greater curvature); C1 and C2 (within 0.5 cm. from the Z line). The biopsy sites are schematically represented in Fig. 1.

**Histopathology and histochemistry**

Biopsy specimens fixed in 10% buffered formalin, were processed, oriented on edge, embedded in paraffin, and cut in sequential 4-μm sections. The median size of biopsy specimens (measured on the glass slide) so obtained and processed in our laboratory is 8 mm in length and 3 mm in thickness. Virtually all specimens included surface epithelium and muscularis mucosae. Slides from each specimen (usually with 8–12 sections) were stained by using our recently developed triple-stain [11]. The procedure, slightly modified since the initial description, is detailed below:

After being placed in a drying oven at 36°C for 2 hr the slides are stained according to the following procedure:
Reagents

1% Uranyl nitrate
Uranyl nitrate  1.0 g
Distilled water  100 mL
(This solution may be reused but discard it after 2 months.)

1% Silver nitrate
Silver nitrate 0.5 g
Distilled water  50 mL
Make fresh each time.

0.04% Silver nitrate
Silver nitrate 0.04 g
Distilled water  100 mL
Make fresh each time.

2.5% Gum mastic  2.5 g
Absolute alcohol  100 mL
(Allow gum mastic to dissolve in the alcohol for 24 hr, then filter the solution until it is clear yellow. Refrigerate at 4°C. This solution may be reused, but do not pour used solution back into the stock bottle. Discard after 2 months.)

2% Hydroquinone
Hydroquinone 1.0 g
Distilled water  50 mL
Make fresh each time.

Reducing Solution
Gum mastic, 2.5% solution  10 mL
Hydroquinone, 2% solution  25 mL
Absolute alcohol  5 mL
Make just before each use, filter through Whatman #4 filter paper, and add 2.5 mL of 0.04% silver nitrate. Do not filter after adding the silver. This solution will have a milky appearance when the gum mastic is added.)

1% Alcian Blue Solution
Alcian blue 8GX  5.0 g
Acetic Acid 3% solution  500 mL
(Adjust the pH to 2.5. Filter and add a few crystals of thymol.)

Procedure

Before staining, place a plastic Coplin jar in a 45°C water bath to heat. Prepare the reducing solution and place in the preheated Coplin jar. (Use only vented plastic Coplin jars in the microwave, be sure that they are loosely capped, and place them inside a loosely closed plastic bag.)

1. Deparaffinize and rehydrate sections to distilled water.
2. Sensitize sections by placing them in room-temperature 1% aqueous uranyl nitrate for 3 min.
3. Rinse slides in distilled water until the possibility of cross contamination is eliminated.
4. Place sections in room-temperature 1% silver nitrate and then heat them in the microwave oven to just below the boiling point—about 42 sec. Do not boil. Remove them from the oven and allow the slides to stand in hot silver nitrate for 10 min.
5. Rinse slides in three changes of distilled water.
6. Rinse in two changes of 95% alcohol.
7. Rinse in two changes of 100% alcohol.
8. Place slides in gum mastic for 5 min.
9. Reduce in reducing solution in a 45°C water bath for 10 to 25 min, or until sections have developed satisfactorily with black spirochetes and a light yellow background.
10. Rinse sections in distilled water to stop reduction.
11. Quickly rinse in 95% alcohol and absolute alcohol, then back to distilled water.
12. Place slides in Alcian blue for 10 min.
13. Rinse in distilled water.
14. Stain in Harris hematoxylin for 8 min.
15. Rinse in tap water.
16. Quick dip in 1% Hydrochloric acid in 70% alcohol.
17. Running water....wash well.
18. Ammonia water, 0.25% or lithium carbonate, 0.5%....3 min.
19. Tap water, two changes.
20. Eosin...5 min or eosin-phloxine.
21. 95% alcohol, two changes.
22. Absolute alcohol, three changes.
23. Xylene, three changes.
Let slides remain in last container until cover slips are applied.

This stain is allowing the simultaneous visualization of *H. pylori*, the gastric mucosal morphology, and the inflammatory infiltrate (Fig. 2). The numbers of *H. pylori*, neutrophils, eosinophils, and mononuclear cells were graded as previously described [8] on a scale ranging from 0 (=absent or normal) through 5 (equivalent to very large numbers or complete obliteration). Intestinal metaplasia was reported as the percentage of intestinal-type epithelium (defined as epithelium containing goblet cells

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Fig. 2. The triple stain allows the distinct visualization of *H. pylori* (fovea on the right) while permitting the observation of all histological features of the gastric mucosa, such as intestinal metaplasia (fovea on the left) and the inflammatory infiltrate.
stained by Alcian Blue at pH 2.5) present on the biopsy. To evaluate the relative contribution of each inflammatory component to the picture of gastritis, we also used a “gastritis index”, calculated by adding the scores for neutrophils, eosinophils and mononuclear cells and dividing the sum by three.

The pathologist had no previous knowledge of the identity of the subjects or their clinical condition.

Data analysis

All scores were entered into a data base and analyzed by using the PC!info Data Management/Analysis System (Retriever Data Systems, Seattle, Washington). Because most data were normally distributed, parametric statistics (ANOVA, Newman-Keuls and Tukey tests, Student’s t-test) and regression analysis were adequate in most cases. Statistical significance of differences and relationships was determined by p values < 0.05.

III. Results

H. pylori infection

Fig. 3 represents graphically the H. pylori score in the four groups of subjects. Non-infected normal volunteers had, by definition a zero score. Infected subjects without ulcer disease had a score of 3.3±0.1 in the antrum and 3.0±0.2 in the corpus. Gastric ulcer patients had the same score of 2.8±0.2 in the antrum and in the corpus; patients with duodenal ulcer had an antral score of 3.3±0.1 and a score in the corpus of 2.5±0.2. The differences in H. pylori density among the three groups of infected patients were not statistically significant. The difference in bacterial density in the antrum and corpus was not significant either in non-ulcer infected patients nor in gastric ulcer patients. In contrast, the corpus of patients with duodenal ulcer had a significantly lower bacterial density than the antrum of the same patients (p<0.01) and than the corpus of infected non-ulcer patients (p<0.05). Although the score was also lower than that of the corpus of gastric ulcer patients (2.5±0.2 vs. 2.8±0.2), this difference did not attain statistical significance.

Gastritis index

Subjects with no evidence of H. pylori infection and no gastrointestinal symptoms had a score of 0 for neutrophils and mononuclear cells in both antrum and corpus. The numbers of eosinophils showed considerable variations in some of the volunteers, resulting in a mean score of 0.4±0.3. The resulting gastritis index for all these normal subjects was, however, 0 (Fig. 4). The most severe inflammatory responses, expressed as the gastritis index, were found in infected subjects without ulcer disease (3.0±6.0 in the antrum and 2.2±0.8 in the corpus); patients with gastric ulcer showed very similar scores (2.9±0.3 in the antrum and 2.1±0.4 in the corpus). In sharp contrast, patients with duodenal ulcer had a significant difference between antrum and corpus (2.5±0.3 in the antrum versus 1.1±0.2 in the corpus; p<0.001).

Intestinal metaplasia

Intestinal metaplasia was completely absent from non-infected subjects. The infected patients in the other three groups had a wide variation in the extent of intestinal metaplasia, resulting in large standard deviations. Patients with non-ulcer gastritis had scattered foci of intestinal metaplasia in both antrum (2.3±1) and corpus (3.1±3); patients with duodenal ulcer had no metaplasia in the corpus, and only foci in the corpus (3.8±2).
trast, most gastric ulcer patients had extensive areas of intestinal metaplasia in the antrum (24.1 ± 8) and, to a much lesser extent, in the corpus (4.2 ± 1.9).

IV. Discussion

This study shows that by using a combined staining technique that allows the simultaneous detection of *H. pylori* and the gastric morphology, it is possible to directly evaluate the relationship between *H. pylori* and the inflammatory cells at the level of the gastric mucosa.

This preliminary study, involving a relatively small number of subjects in each category has, nevertheless, provided some interesting results. First, in spite of the extensive biopsy sampling, we did not detect intestinal metaplasia or atrophic gastritis in any of the non-infected volunteers. This indicates that in our population *H. pylori* infection appears to be a necessary condition for the development of intestinal metaplasia. Because, however, the majority of infected subjects do not have intestinal metaplasia, it is likely that other factors must act on a background of chronic active gastritis to induce the lesions that lead to metaplastic changes.

In all three groups of infected patients, irrespective of the concurrent presence of peptic ulcer, both the intensity of gastritis and the density of bacterial infection were more severe in the antrum than in the corpus. In this respect, our results are consistent with those of other topographical studies [21]. A true pattern of “antral predominant gastritis,” however, was present only in patients with duodenal ulcer. This confirms previous observations that a structurally intact corpus is necessary to develop a duodenal ulcer [5, 20, 22]. It is interesting to note that, while in these patients there was a considerable difference in the gastritis index between antrum and corpus, the difference in the bacterial intensity in the two gastric compartments was much less distinct. This suggests that the mucosa of the corpus of patients who develop duodenal ulcer not only is less hospitable for *H. pylori*, but also develops a lesser inflammatory response against the infecting organisms.

One important consideration when evaluating these results is that patients with *H. pylori* gastric and no current evidence of ulcer do not necessarily represent a static group: while some may indeed have had and never develop an ulcer, it is quite possible that some of these patients had ulcers in the past that were not diagnosed and that some will develop them in the future. Therefore, all that can be said about these subjects is that they had no evidence of ulcer disease at the time of the study.

With respect to the staining techniques, two issues need to be addressed. One regards the specificity of the stain, the other the presence of bacteria in the gastric mucus. Silver stains are clearly not specific for *Helicobacter* spp. All spirochetes and many gram-negative and -positive bacteria take up our stain; therefore, one must consider the possibility that we have included in our evaluation of infection and bacterial load some non-*H. pylori* organisms. We believe this is extremely unlikely for two reasons. First, the stomach is only rarely infected by other bacteria, and in these rare situations (e.g., syphilitic or phlegmonous gastritis) both the clinical manifestations and the pattern of infection are very distinct from those of *H. pylori* infection. Second, our stain permits a very detailed view of *H. pylori*: its characteristic spiral structure and the presence of two polar darker dots make it virtually impossible to confuse with other organisms (Fig. 5). As for other *Helicobacter* species that may infect humans (e.g. *Helicobacter felis*), their elongated tightly spiral shape will allow a rapid differentiation from *H. pylori*. Although more specific, the sensitivity of immunohistochemical techniques has not been reliably evaluated. In our laboratory, we often find difficult to separate small particles of non-specific immunoperoxidase staining from individual bacteria. Thus, we limit the performance of immunohistochemical studies to those rare cases in which the contribution of a more specific test appears necessary.

The issue of bacteria in the mucus is important. Although our careful handling of gastric biopsy specimens limits the amount of damage to the surface mucus when they are removed from the biopsy forceps, fixation in formalin greatly reduces the amount of mucus on the mucosal surface. Therefore, we may have consistently underestimated the numbers of *H. pylori*. Only recently have we become aware of the combined staining techniques developed by Ota and Katsuyama [18], and are currently attempting to adapt our triple stain to Carnoy’s-fixed gastric biopsies. The inclusion of *H. pylori* present in the mucus layer in the evaluation of the bacterial density may provide new insights into the distribution of this infection and, consequently, into some of the complex pathogenetic mechanisms of gastritis.

Fig. 5. *H. pylori* stain with two characteristic darker polar dots. Their spiral shape is also readily visible.
V. References


