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Helicobacter pylori Causes Hyperproliferation of the Gastric Epithelium: Pre- and Post-Eradication Indices of Proliferating Cell Nuclear Antigen

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Objective: To assess the effect of *Helicobacter pylori* (HP) eradication on the proliferation of the gastric epithelium by the expression of the proliferating cell nuclear antigen (PCNA). Methods: Alcohol-fixed gastric biopsies taken before and after treatment for HP were immunostained with the PC-10 anti-PCNA monoclonal antibody and the labeling index was determined with an image analysis system. Results: The mean PCNA-labeling index (LI) of 16 patients who remained HP positive did not change significantly (18.95 ± 1.71 on first visit vs. 17.96 ± 1.91 on second visit, mean ± SEM). The mean PCNA LI of 31 patients who cleared HP was reduced significantly (19.95 ± 1.77 on first visit *vs.* 14.13 ± 1.29 on second visit, $p < 0.001$). Patients who were positive for HP at both first and second visit showed a significantly higher PCNA LI than normal control biopsies (13.05 ± 1.70) ($p < 0.05$). Conclusions: These results indicate that the gastric mucosa infected with HP is in a state of hyperproliferation. Patients who cleared HP showed a significant histopathological improvement, reflected in the reduction in number of polymorphonuclear and mononuclear cell infiltrates, and a reduction in the amount of superficial mucosa damage. Prolonged hyperproliferation of the gastric epithelium exerted by HP infection could be a major factor for human gastric carcinogenesis.

INTRODUCTION

Gastritis associated with *Helicobacter pylori* (HP) infection is among the most frequent chronic infectious diseases in the world. Its estimated prevalence in adults varies from approximately 30% to near 100% in different populations (1–3). According to some authors, the syndrome most frequently related to HP infection is non-ulcer dyspepsia (4, 5). The bacterium is also considered a contributing cause of peptic ulcer and gastric carcinoma (2, 6–11).

Because the bacterium colonizes the gastric lumen but does not invade the tissues, the mechanisms of tissue damage are the subject of scientific scrutiny. Cellular damage due to bacterial cytotoxin (12) and to ammonia produced by its potent urease have been implicated (13). The mechanism of its putative carcinogenic effect is unknown. This report explores the hypothesis that excessive cell replication, a factor of major relevance in carcinogenesis (14, 15), may be caused in the gastric mucosa by HP infection. Cell replication is assessed in this study by the effect of HP eradication on the expression of proliferating cell nuclear antigen (PCNA).

MATERIALS AND METHODS

Fifty-eight patients referred for upper gastrointestinal endoscopy at Charity Hospital in New Orleans, with a clinical and endoscopic diagnosis of non-ulcer dyspepsia, were included in this study. Each agreed to participate in a longitudinal study of the effects of HP on gastric histopathology, approved by the Louisiana State University Institutional Review Board. Eleven patients who were found to have normal gastric mucosa were negative for HP. These subjects had only one visit and were used as controls. The rest of the patients had gastroscopic biopsies positive for HP on rapid urease test (16) and were invited to participate in the study. The subjects were assigned treatments after the first visit as follows: 13 received no specific treatment for HP, 23 received triple therapy consisting of: *1)* bismuth subsalicylate (262 mg *qid* for 28 days), *2)* metronidazole (500 mg *qid* on days 18–28), and *3)* amoxycyllin (500 mg *qid* on days 8–24); and 11 patients received triple therapy with nitrofurantoin (100 mg *qid* on days 8–17) instead of amoxycyllin. Endoscopies and biopsies were repeated on the second visit as follows. In those who received triple therapy, the mean time from termination of treatment to second biopsy was 3 days (range 12 h to 23 days), with a median of 1 day. In this group, 10 patients had a second biopsy 12 h after finishing treatment, and 24 patients had a second bi-

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opsy 36 h or more after finishing treatment. Patients who received no specific treatment for HP had a second biopsy after a mean of 147 days (range 56–226 days), and a median of 168 days.

Five antral biopsies and one corporal biopsy were taken on each visit. One antral biopsy, from the lesser curvature, was used for the rapid urease test for HP. Two antral biopsies (greater and lesser curvature) were fixed in 95% alcohol, and two (greater and lesser curvature) were fixed in 10% buffered formalin. The corporal biopsy was also fixed in 10% buffered formalin. Tissues were dehydrated and paraffin embedded, sectioned at 3 μm and placed on poly-L-lysine (Sigma Chemical Co.) -coated glass slides. The formalin-fixed tissues were stained with hematoxylin and eosin, periodic acid-Schiff-Alcian blue (PAS-AB) pH 2.5 (17), and the Steiner modification of Warthin-Starry staining for HP (18).

The 95% alcohol fixed tissues were immunostained for PCNA with PC10 monoclonal antibody (Dako Corporation), by a microwave-assisted streptavidin-biotin peroxidase method, and the Fisher Capillary System (Fisher Scientific). This method allowed us to immunostain 60 slides at the same time, under the same conditions for antibody incubation. Briefly, sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 s in a microwave oven at high power (1440 watts). After washing in deionized water, sections were boiled 6 min in deionized water in the microwave oven at high power and allowed to stand for 3 min (19). Sections were placed in the Capillary System slide holder and washed in phosphate-buffered saline (PBS) (pH 7.2) with 0.01% Tween 20 (Sigma) 3 times for 10 s, then were drained over a thick paper pad. All the antibody incubations were done in the microwave oven at 30% power and allowed to stand for 3 min. PC10 monoclonal antibody was incubated for 14 min. Secondary biotinylated goat antimouse immunoglobulins (Dako) was incubated for 7 min. Streptavidin-peroxidase complex (Dako) was incubated for 7 min. Diaminobenzidine (Sigma) was used at 0.05% in PBS for 10 min at room temperature. Sections were lightly counterstained with Harris hematoxylin, dehydrated, and mounted. Negative controls included substitution of primary antibody with PBS or normal mouse serum.

A Joyce-Loebl Magiscan (London, England) image analysis system was used to assess the PCNA nuclear labeling. PCNA was evaluated in areas corresponding to gastric antrum glandular necks at ×400, using a static 560-nm filter. Seven glandular necks were selected at random in each specimen to ensure a minimum of 250 total nuclei. System thresholding was adjusted so only those nuclei stained a strong dark brown were counted as positive. Then, the threshold was adjusted to count the total nuclei in the selected areas. The mean number of nuclei counted per specimen was 508 (range 267–752, median 526). The PCNA labeling index (PCNA LI) was defined as a percentage of the PCNA-positive nuclei over the total nuclei in glandular neck regions.

Biopsies were diagnosed histopathologically according to previously defined criteria (20) in diffuse antral gastritis (DAG) and multifocal atrophic gastritis (MAG) on formalin-fixed and hematoxylin and eosin-stained sections of corpus and antrum. DAG is characterized by a dense infiltrate of lymphocytes and plasma cells occupying the full thickness of the antral mucosa. The infiltrate expands the lamina propria, separating the gastric glands, and lymphoid follicles may be prominent; this entity is most often related to the duodenal ulcer syndrome. MAG corresponds to multifocal glandular atrophy accompanied by an inflammatory mononuclear infiltrate, and is located in antrum and body. Intestinal metaplasia is often found associated with MAG. A semiquantitative assessment of the histopathologic changes was made by two observers; discrepancies were resolved by consensus after joint review. The presence of polymorphonuclear and mononuclear cells in the lamina propria and glands was evaluated as 0 = normal or very rare, 1 = a few or equivocal, 2 = moderate number, and 3 = marked increase. Damage to superficial and foveolar epithelia, along with mucus depletion, was evaluated in both hematoxylin and eosin and PAS-AB, and given the following scores: 0 = normal superficial mucosa, 1 = mild superficial mucosa damage, 2 = moderate superficial mucosa damage, and 3 = marked superficial mucosa damage. The presence of HP was scored accordingly, 0 = negative, 1 = small number, 2 = moderate number, and 3 = abundant. A global histological score, adding all the previous parameter scores except for HP, was used for comparison.

All of the slides were evaluated blindly, without previous knowledge of the histological diagnosis, treatment, or treatment outcome for HP. PCNA LI was evaluated only in non-metaplastic areas.

Student's *t* test (two-tailed) was used to evaluate pre- and post-treatment differences in PCNA LI. Comparison of histopathological scores was performed by Wilcoxon's rank test (two-tailed).

RESULTS

The study included 39 females and 19 males, with a mean age of 47.7 yr (range 20–70). Histological diagnosis included 40 patients with MAG, seven with DAG, and 11 normal gastric biopsies used as controls.

Immunostaining of PCNA, with a diffuse granular pattern, was localized in the nuclei. The staining intensity was variable. In the normal cases, strong labeling was observed only in the glandular necks (Fig. 1A).

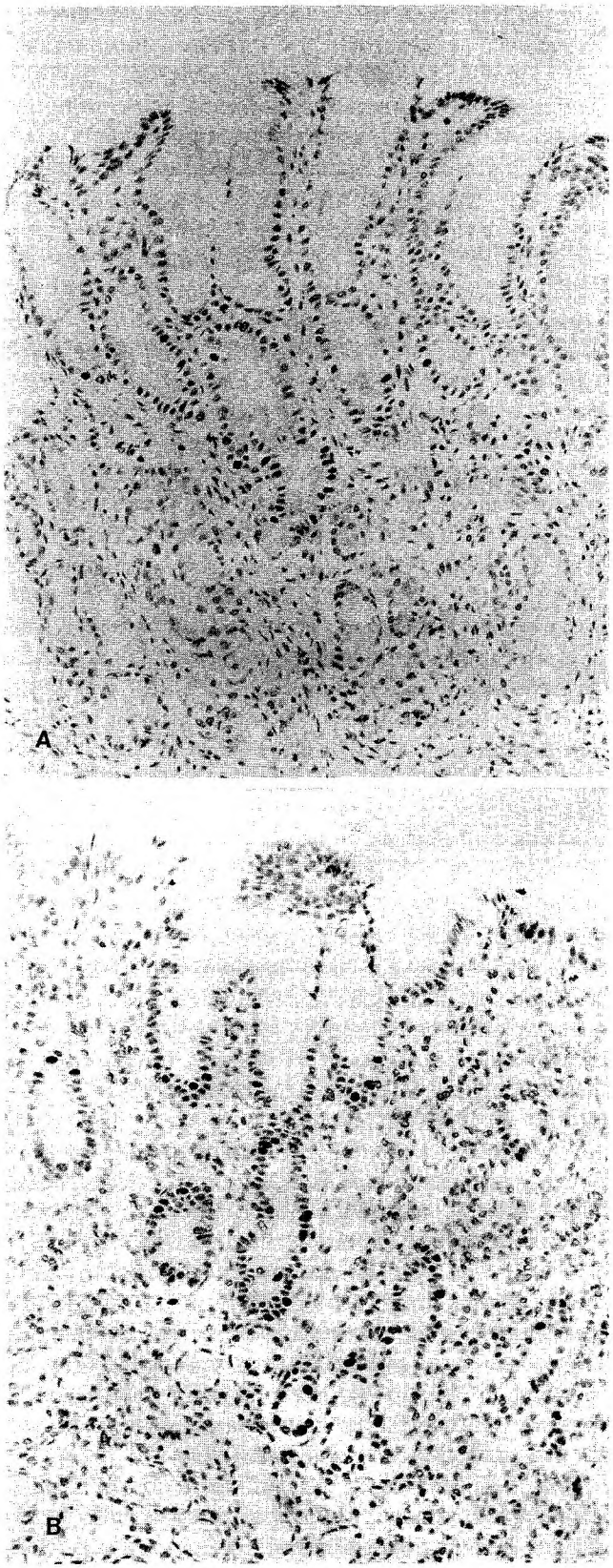

FIG. 1. PCNA immunostaining of the gastric mucosa. *A*, normal gastric mucosa expressing PCNA in the nuclei of cells of the glandular necks (×200). *B*, MAG, showing an increased number of PCNA-positive nuclei and extension to superficial and deep areas of the mucosa (×200).

Some of these cases had weak staining in a few cells of the superficial mucosa. In chronic gastritis, strong nuclear PCNA immunostaining was observed mainly in glandular necks (Fig. 1B), but in some cases, few stained nuclei were seen in the deep or superficial areas of the mucosa. The PCNA stain was evaluated only in glandular necks. The few positively stained nuclei in other layers were insufficient for adequate evaluation.

The 11 patients with normal gastric histology and negative for HP showed a mean PCNA LI of 13.05 ± 1.70 (Table 1). On the second visit, of the 47 patients initially positive for HP, 31 cleared and 16 persisted with the infection. In patients who remained HP positive, the PCNA LI did not change significantly (Table 1). The mean PCNA LI for those who cleared HP decreased significantly ($p < 0.001$). Patients who remained positive for HP, on both the first and second visit, showed a significantly higher PCNA LI than normal control biopsies ($p < 0.05$). However, patients who cleared HP infection showed a significantly higher PCNA LI only on the first visit, in relation to normal gastric mucosa ($p < 0.01$).

According to histopathological diagnosis, patients with MAG, whose HP cleared, showed a significant reduction in PCNA LI (n = 19, 22.10 ± 2.61 *vs.* 15.09 ± 1.33, $p < 0.05$), and those that did not clear showed no difference (n = 16, 20.71 ± 2.65 *vs.* 19.51 ± 1.97). In DAG, five patients cleared HP and showed a tendency to maintain similar PCNA LI (17.85 ± 3.52 *vs.* 16.90 ± 5.69); two patients did not clear, but were not sufficient for statistical analysis.

Histopathological parameters were evaluated by the system of scores described above. In patients who cleared HP, there was a significant reduction of the inflammatory infiltrate, reflected in the scores for polymorphonuclear and mononuclear cell infiltration ($p < 0.001$) (Table 2). A significant improvement in the mucus production ($p < 0.001$) was observed in those patients who cleared HP. These improvements after HP clearance were reflected in the significant reduction of the global histopathological score ($p < 0.001$). Histopathological parameters in patients who remained positive did not change significantly.

Table 3 displays the PCNA LI on the second visit, according to the histologic parameters studied and HP

TABLE 1
PCNA Labeling Index According to Study Results*

	First Visit	Second Visit	*p*
HP cleared (n = 31)	19.95 ± 1.77	14.13 ± 1.29	<0.001
HP persisted (n = 16)	18.95 ± 1.71	17.96 ± 1.91	NS†
Nl. histology, HP −ve (n = 11)	13.05 ± 1.70		

* Mean ± SEM.
† NS, not statistically significant.

TABLE 2
Comparison of Scores for Histological Parameters and Helicobacter pylori Status

Parameter	HP Cleared (n = 31)			HP Persisted (n = 16)		
	First visit	Second visit		First visit	Second visit	
	Median score		*p*	Median score		*p*
Sup. mucosa dam.	2	0	<0.001	1	2	NS
PMN cells	2	0	<0.001	2	2	NS
MN cells	2	1	<0.001	2	2	NS
Histol. score	7	3	<0.001	6	7	NS

Abbreviations: Sup. mucosa dam, superficial mucosa damage; PMN, polymorphonuclear; MN, mononuclear; NS, not statistically significant.

TABLE 3
PCNA LI According to Histologic Parameters and HP Status on Second Visit*

	All Observations (n = 58)		HP Positive (n = 16)		HP Negative (n = 31)	
PMN infiltrate						
Absent or mild	(46)	13.91 ± 0.99	(4)	14.53 ± 3.40	(31)	14.13 ± 1.29
Moderate or severe	(12)	19.11 ± 2.26	(12)	19.11 ± 2.26	(0)	
		$p = 0.024$		$p = 0.316$		ISS
MN infiltrate						
Absent or mild	(33)	13.90 ± 1.25	(4)	14.53 ± 3.40	(18)	14.27 ± 1.97
Moderate or severe	(25)	16.41 ± 1.41	(12)	19.11 ± 2.26	(13)	13.93 ± 1.51
		$p = 0.190$		$p = 0.316$		$p = 0.898$
Sup. mucos. dam.						
No depletion	(45)	13.90 ± 0.99	(5)	12.04 ± 2.41	(29)	14.54 ± 1.34
Some depletion	(13)	18.73 ± 2.23	(11)	20.65 ± 2.15	(2)	8.18 ± 0.78
		$p = 0.031$		$p = 0.031$		ISS

Results are expressed as mean ± SEM. Number of patients in parentheses. PMN, polymorphonuclear cells; MN, mononuclear cells; Sup. mucos. dam., superficial mucosa damage; ISS, insufficient sample size.

status. If the whole study group is considered, those patients with a moderate or severe polymorphonuclear cell infiltrate and those with superficial mucosa damage showed a significantly higher PCNA LI. In the whole group, and in those positive for HP, the greater intensity of the mononuclear infiltrate was associated with a tendency to a higher LI, but it did not reach statistical significance. After clearance of HP, the PCNA LI did not differ significantly according to the intensity of mononuclear infiltrate. In the group that cleared HP, both the polymorphonuclear infiltrate and the superficial mucosa damage disappeared, making impossible their independent evaluation.

DISCUSSION

The value of PCNA LI in assessing cell proliferation is supported by studies of carcinoma of the stomach showing a good correlation with S + G_2M phase of flow cytometric analysis (21) and with Ki-67 (22). In other systems, PCNA LI has shown a good correlation with other indexes of cell proliferation such as [^{3}H]thymidine (23, 24), and bromodeoxyuridine (BrdUrd) (24, 25). PCNA is mainly expressed in late G1 phase, during the S phase and early G2 (26), and it can be detected at low levels in cells during the cell cycle. PCNA LI, compared with BrdUrd and flow cytometry, has shown a higher percentage of proliferating cells (22, 23, 27). One explanation offered for these results is the long half-life of PCNA (about 20 h) (28). Image analysis thresholding has allowed us to select only those nuclei that are strongly positive, which has been reported to have a better correlation with BrdUrd and flow cytometry cell proliferation indices (29, 30). PCNA reportedly had a better correlation with [^{3}H]thymidine labeling when alcohol-fixed tissues were used (23, 27, 29, 30).

Cell proliferation studies in chronic gastritis have shown a high number of proliferating cells (31, 32), consistently higher than the normal gastric mucosa. There have been similar findings on colonic mucosa, in which a state of hyperproliferation is present in precancerous conditions such as familial polyposis, adenomas, and ulcerative colitis (33–36).

Our study shows a significant reduction of PCNA-labeled cells, indicative of cell replication, after clearance of HP. The post-clearance indices are very close to those of the normal gastric mucosa. These results indicate that the infected gastric mucosa is in a state of hyperproliferation.

Histopathological parameters were improved after

HP eradication in our study. Both acute and chronic inflammatory infiltrates were reduced in significant numbers. Superficial mucosa damage and mucus depletion were significantly attenuated after HP clearance. These changes corroborate those observed by others (37–40) and confirm the damage inflicted by HP on the gastric mucosa. Changes in the chronic (lymphocytic) infiltrate need to be studied for a longer term.

Given the chronicity of the gastritis, it is probable that the state of hyperproliferation is present for many years in most patients. Because HP infection increases gastric cancer risk, this situation fits the hypothesis proposed by Ames and others (41, 42) that in a hyperproliferative state, endogenous ("spontaneous") mutations may induce genetic lesions which accumulate and eventually might lead to neoplastic transformation (43, 44). *Helicobacter*-associated gastritis provides a model to use to examine this hypothesis, because persistence of hyperproliferation over a long period of time is associated with an increased risk of carcinoma (2, 8, 6, 45, 46). According to the Ames hypothesis, endogenous mutations *per se*, without external mutagens or carcinogens, may be sufficient to induce neoplastic changes in tissues in a state of hyperproliferation (42).

In populations at high risk of gastric carcinoma, the prevalence of *Helicobacter*-associated gastritis is very high (2, 8, 6, 45, 46). The type of gastritis predominant in such populations is the so called multifocal atrophic gastritis (MAG), as it happens in the New Orleans blacks, who predominate in our study (12). Mucosal atrophy is followed by intestinal metaplasia and, eventually, dysplasia and carcinoma (47). *Helicobacter* infection is very prevalent and severe in such populations, which strongly suggests a causative role for the bacteria in carcinogenesis (9).

HP, however, is also prevalent in DAG, which is the predominant type of gastritis in populations at low gastric cancer risk, such as the white middle-class Americans (9). DAG is also the predominant type of gastritis seen in children. Recent studies in Costa Rica compared the prevalence of gastritis and *Helicobacter* infection in children from the central plateau (who have some of the highest gastric cancer risks on record), with those from the coastal lowlands, where the gastric cancer risk is low (48). In both populations the prevalence of HP-associated gastritis is very high: around 70% in children of 7–12 yr of age. Because the gastritis in the coastal lowlands is not associated with higher cancer risk, it follows that HP is not a sufficient cause of gastric cancer. Other factors, presumably present in the plateau highlands, are needed and may probably be more effective in the presence of the hyperproliferative state brought about by HP infection. The same pattern of high prevalence of HP-associated chronic gastritis but low gastric cancer risk is observed in Africa (3). These observations suggest that endogenous mutations *per se* are not sufficient to increase the cancer rates to epidemic proportions.

Our studies indicate that prolonged hyperproliferation of the gastric epithelium is a major mechanism by which HP exerts a contributing causal influence in human gastric carcinogenesis.

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