

## *Helicobacter pylori* cagA<sup>+</sup> Strains and Dissociation of Gastric Epithelial Cell Proliferation From Apoptosis

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**Background:** Infection with *Helicobacter pylori* induces chronic gastritis in virtually all infected persons, and such gastritis has been associated with an increased risk of developing gastric cancer. This risk is further enhanced with cagA<sup>+</sup> (positive for cytotoxin-associated gene A) *H. pylori* strains and may be a consequence of induced gastric cell proliferation and/or alteration in apoptosis (programmed cell death) in the gastric epithelium. **Purpose:** To determine whether the *H. pylori* cagA genotype and another virulence-related characteristic, the vacA (vacuolating cytotoxin A) s1a genotype, differentially affect epithelial cell proliferation, apoptosis, and the histologic parameters of inflammation and injury, we quantitated these characteristics in infected and uninfected persons. **Methods:** Fifty patients underwent upper gastrointestinal endoscopy, and biopsy specimens were taken. Apoptotic cells in the specimens were quantitated after terminal deoxynucleotidyl transferase labeling of DNA fragments with digoxigenin-deoxyuridine triphosphate; epithelial cell proliferation was scored by immunohistochemical analysis of the proliferation-associated antigen Ki-67. Antibodies directed against *H. pylori* and CagA protein were measured in the serum of patients by means of enzyme-linked immunosorbent assays. Analysis of *H. pylori* genomic DNA, by use of the polymerase chain reaction, was performed to determine the cagA and vacA genotypes. Acute and chronic inflammation, epithelial cell degeneration, mucin depletion, intestinal meta-

plasia, glandular atrophy, and vacuolation were each scored in a blinded manner. Reported *P* values are two-sided. **Results:** Persons harboring cagA<sup>+</sup> strains (n = 20) had significantly higher gastric epithelial proliferation scores than persons infected with cagA<sup>-</sup> strains (n = 9) or uninfected persons (n = 21) (*P* = .025 and *P* < .001, respectively), but the difference in cell proliferation between the latter two groups was not statistically significant. The number of apoptotic cells per 100 epithelial cells (apoptotic index) in persons infected with cagA<sup>+</sup> strains was lower than in persons infected with cagA<sup>-</sup> strains (*P* = .05). Apoptotic indices in the cagA<sup>+</sup> group were similar to those in the uninfected group (*P* = .2). Epithelial cell proliferation was significantly correlated with acute gastric inflammation, but only in the cagA<sup>+</sup> group (*r* = .44; *P* = .006). The cagA<sup>+</sup> and vacA s1a genotypes were found to be concordant, confirming the close relationship between these virulence-related genotypes. **Conclusions:** Gastric mucosal proliferation was significantly correlated with the severity of acute gastritis in persons infected with cagA<sup>+</sup> vacA s1a strains of *H. pylori*. This increased proliferation was not accompanied by a parallel increase in apoptosis. **Implications:** Increased cell proliferation in the absence of a corresponding increase in apoptosis may explain the heightened risk for gastric carcinoma that is associated with infection by cagA<sup>+</sup> vacA s1a strains of *H. pylori*. [J Natl Cancer Inst 1997;89:863-8]

*Helicobacter pylori* infection results in chronic gastritis in virtually all persons who harbor the organism, and some infected persons later develop atrophic gastritis and adenocarcinoma affecting the distal stomach (1-6). Development of most gastric adenocarcinomas is believed to follow a series of defined histologic steps from normal gastric mucosa to chronic gastritis, to atrophic gastritis and intestinal metaplasia, and, finally, to dysplasia and neoplasia (7). It has been postulated that *H. pylori* plays a causative role at the early phases in this chain of malignant progression. However, only a small percentage of persons carrying *H. pylori* develop neoplasia; enhanced can-

cer risk may be related either to differences in host response to the bacteria, to differences in expression of specific bacterial products, or both. Host mechanisms that may be linked to neoplastic transformation include increased cellular turnover, damage to the gastric mucosa by reactive oxygen metabolites, and reduction in the gastric juice concentration of ascorbic acid, a potent antioxidant (7,8).

*H. pylori* strains possessing the cytotoxin-associated gene cagA are associated with significantly increased risk for the development of atrophic gastritis and gastric adenocarcinoma (9-11). A second locus of heterogeneity among *H. pylori* strains concerns vacA, which encodes the vacuolating cytotoxin. *H. pylori* strains of the vacA s1a subtype are usually strongly toxigenic, and this subtype is tightly linked with the presence of cagA, although exceptions occur (12).

Increased cellular proliferation rates are characteristic in malignant tissue, and gastric epithelial hyperproliferation in *H. pylori*-infected persons has been reported (13,14); in one study (14), the degree of proliferation was directly associated with severity of mucosal neutrophilic infiltration. Infection with cagA<sup>+</sup> *H. pylori* strains, in comparison with cagA<sup>-</sup> strains, also is linked with higher acute inflammatory scores (15,16), suggesting that these strains could preferentially induce epithelial cell proliferation by stimulating inflammatory mediators. However, to main-

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tain tissue integrity, heightened rates of cell production should be matched by increased rates of cell loss. In chronic *H. pylori* infection, there is a notable lack of epithelial necrosis (17,18), suggesting that other forms of cellular demise such as apoptosis may be induced.

Apoptosis is a form of programmed cell death that consists of a tightly regulated series of energy-dependent molecular events (19). Malignant transformation may occur, in part, because of a failure to activate apoptosis and delete cells with genetic damage. We sought to determine whether *H. pylori* isolates predicted to differ in premalignant potential differentially alter gastric epithelial cell proliferation and apoptosis. Therefore, we quantitated epithelial proliferation and apoptosis in vivo in *H. pylori*-infected and uninfected persons and examined these processes in relation to *cagA* and *vacA* genotypes and histologic parameters of inflammation and injury.

## Methods

### Clinical Specimens

Patients scheduled for upper endoscopy were enrolled prospectively from the Nashville Department of Veterans Affairs Medical Center Gastroenterology Clinic from May 1993 through October 1996. The study protocol was approved by the Vanderbilt University and the Nashville Department of Veterans Affairs Institutional Review Board. Patients were excluded from this study if they had a history of nonsteroidal anti-inflammatory drug ingestion, were receiving steroids or other immunomodulating drugs, were abusing alcohol or illicit drugs, had an active infection or cancer, had taken antimicrobial agents within the prior 2 weeks, were hepatitis B sAg or human immunodeficiency virus positive, or had active gastrointestinal bleeding. After obtaining a thorough medication history and a written informed consent, blood was obtained to measure the serum-specific immunoglobulin G response to *H. pylori* whole-cell antigen and CagA protein by previously validated enzyme-linked immunosorbent assays (10,20). Upper endoscopy was performed and endoscopic findings were recorded. Four gastric antral biopsy specimens from a single site 5 cm proximal to the pylorus were obtained from each patient: two were formalin fixed for analysis of proliferation, apoptosis, and histologic studies; and two were cultured for *H. pylori* under microaerobic (less aerobic than normal atmosphere) conditions, as previously described (16).

### Histology

Hematoxylin-eosin-stained paraffin sections of biopsy specimens were examined without knowledge of the experimental results by a single experienced pathologist (K. T. Tham). Histologic parameters included assessment of the degree of neutrophil and mononuclear cell infiltration, cellular atrophy,

intestinal metaplasia, epithelial cell surface degeneration, mucin depletion, and epithelial cell vacuolation, each graded on a scale of 0-3, as previously described (16). Acute inflammation was defined as the degree of epithelial and stromal neutrophil infiltration and was scored as follows: grade 0—no polymorphonuclear cells (PMNs) present; grade 1—focal mild neutrophil infiltration (<10 PMN/high-powered field [HPF]); grade 2—focal dense neutrophil infiltration (10-20 PMNs/HPF); or grade 3—diffuse and dense PMN infiltration (>20 PMNs/HPF). Chronic inflammation was defined as the degree of mononuclear cell infiltration and was scored as follows: grade 0—no infiltration; grade 1—mild inflammation (slight increase in mononuclear cells with no lymphoid follicles); grade 2—moderate inflammation (dense but focal mononuclear inflammatory cell infiltrate with no lymphoid follicles); or grade 3—severe inflammation (dense and diffuse mononuclear inflammatory cell infiltrate with or without lymphoid follicles). The modified Giemsa stain was used for histologic identification of *H. pylori* (21).

### Bacterial Culture and Polymerase Chain Reaction (PCR) of Genomic DNA From Clinical Isolates

Gastric biopsy specimens were placed immediately in normal saline at 4 °C and coarsely homogenized in 250  $\mu$ L normal saline with the use of a tissue grinder (Micro Kontes, Vineland, NJ). Fifty microliters was plated onto Trypticase soy agar with 5% sheep blood and incubated for 96 hours under microaerobic conditions, as described previously (16). To genotype *H. pylori* isolates, genomic DNA was prepared (22) and PCR for *cagA* and *vacA* signal sequence type was performed, as described previously (12,16). Briefly, PCR was performed in a total volume of 50  $\mu$ L, containing buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M (each) deoxynucleotides, 2 U AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 100 ng genomic DNA as template. PCR amplification of *cagA* used previously described primers F1 (5'-GATAACAGGCAAGCTTTTGAGG-3') and B1 (5'-CTGCAAAAGATTGTTTGGCAGA-3') (23). Forward primers for typing *vacA* signal sequences included: s1a (5'-GTCAGCATCACACCGCAAC-3'); s1b (5'-AGCGCCATACCGCAAGAG-3'); and s2 (5'-GCTAACACGCAAAATGATCC-3'). The same reverse primer was used for carrying out PCR for each signal sequence: (5'-CTGCTTGAATGCCCAAAC-3'). All primers were used at a final concentration of 0.5  $\mu$ M. Each reaction mixture was amplified for 35 cycles as follows: 1 minute at 94 °C, 1-minute primer annealing at 55 °C (for *cagA*) or 52 °C (for *vacA*), and 2 minutes at 72 °C. After the 35th cycle, extension was continued for another 7 minutes. The lower limit of detection by PCR by use of these primers is approximately 10 ng genomic DNA. Agarose gel electrophoresis (2% gel at 70 vol for 1 hour) followed by ethidium bromide staining was performed on an aliquot from each PCR.

### Proliferation and Apoptosis Assays

For each patient, proliferation and apoptosis scores from two gastric antral biopsy specimens were averaged. A single pathologist (K. T. Tham) blinded to *H. pylori* infection status quantitated gas-

tric epithelial cell proliferation in formalin-fixed antral biopsy specimens by use of an immunoperoxidase stain (BioGenex Laboratories, San Ramon, CA) for Ki-67, a cell proliferation-associated antigen (24). Staining was scored 1-3 as follows: grade 1—up to one third of the isthmus and neck cells was stained and the length of the proliferative zone did not exceed one half of the pit length; grade 2—one third to two thirds of the isthmus and neck cells was stained and the length of the proliferative zone was approximately three fourths of the pit length; and grade 3—more than two thirds of the isthmus and neck cells was stained and the length of the proliferative zone equaled the pit length.

Epithelial apoptosis was quantitated in situ from the same formalin-fixed biopsy specimens by use of terminal uridine deoxynucleotidyl nick end-labeling (TUNEL), as previously described (17), but digoxigenin-11-deoxyuridine triphosphate was used at a concentration of 0.4 nM. At least 500 epithelial cells were counted in each of two sections per biopsy specimen, and the mean number of positive cells per 100 total epithelial cells was expressed as the apoptotic index (%). Apoptotic cells were quantitated by a single observer (S. Wang) who was unaware of proliferation scores, infection status, or genotype of the infecting *H. pylori* isolate. The apoptosis/proliferation ratio was determined by dividing apoptotic cellular index by proliferation score.

### Statistics

Two-tailed statistical tests were used to evaluate the data. The nonparametric Mann-Whitney *U* test was used for pairwise comparisons while the Kruskal-Wallis test was used for overall comparisons between patient groups. Differences between scores of inflammatory parameters, proliferation, and apoptosis in the same patients were compared by use of the linear regression analysis. Significance was defined as  $P \leq .05$ .

## Results

### Characteristics of *H. pylori* Strains

In total, 50 persons were enrolled: 21 were uninfected and 29 persons were infected with *H. pylori* as determined by serology, histology, and/or culture. Of the 29 infected patients, 20 (69%) harbored *cagA*<sup>+</sup> strains and nine (31%) were infected with strains that lacked *cagA* as determined by both serology and PCR of genomic DNA. The *vacA* signal sequence type could be determined for 23 of these strains; 13 (57%) were s1a, four (17%) were s1b, and six (26%) were s2. Of the 23 evaluable strains, *cagA* and *vacA* s1a signal type were concordant (both positive or negative) in 19 (83%) of the 23 strains ( $P = .006$ ), confirming the close relationship noted previously (12) between these virulence-related genotypes.

## Measurement of Epithelial Cell Proliferation and Apoptosis in Uninfected Patients and Patients Infected With *H. pylori*

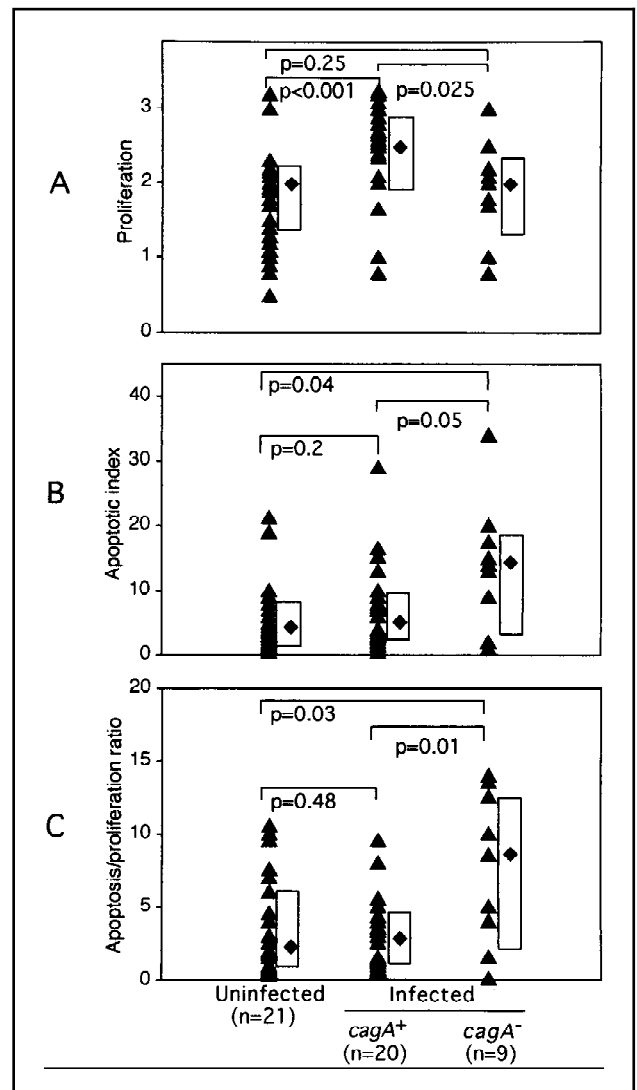
Since infection with *H. pylori* *cagA*<sup>+</sup> strains is linked to a higher risk for development of atrophic gastritis and gastric cancer, we determined whether epithelial cell proliferation and apoptosis were directly associated with *H. pylori* strain characteristics. Persons harboring *cagA*<sup>+</sup> strains had significantly higher antral proliferation scores than either persons infected with *cagA*<sup>-</sup> strains or uninfected persons ( $P = .025$  and  $P < .001$ , respectively) (Fig. 1, A), but there were no significant differences in proliferation between these latter two groups ( $P = .25$ ). A significant difference in proliferation overall was found when the three groups were analyzed together ( $P = .004$ ).

When we examined the cellular apoptotic index in the same three patient groups (Fig. 1, B), the pattern observed was distinct from that for antral epithelial proliferation; persons infected with *cagA*<sup>+</sup> strains had significantly lower apoptotic indices than persons infected with *cagA*<sup>-</sup> strains ( $P = .05$ ), and the *cagA*<sup>+</sup> group did not differ significantly from uninfected persons ( $P = .2$ ). In addition, no significant difference was found overall when the three groups were compared together ( $P = .14$ ). When antral proliferation and apoptosis were examined in relation to *vacA* signal sequence types, results for *s1a* strains paralleled those for *cagA*<sup>+</sup> isolates (data not shown).

### Apoptosis/Proliferation Ratios and Infection With *H. pylori*

We next derived an apoptosis/proliferation ratio that permitted expression of the relationships between epithelial cell growth and loss in each biopsy specimen as a single term. Persons infected with *cagA*<sup>+</sup> strains had significantly lower apoptosis/proliferation ratios than persons infected with *cagA*<sup>-</sup> strains ( $P = .01$ ), and there were no differences in the ratios between uninfected persons and those harboring *cagA*<sup>+</sup> strains ( $P = .48$ ) (Fig. 1, C). A trend toward significance was seen when the three groups were analyzed together ( $P = .097$ ). As before, results for persons infected with *vacA* *s1a* strains reflected those for *cagA*<sup>+</sup> strains. In summary, the results show that infection with

Fig. 1. Proliferation scores (A) are higher but apoptosis scores (B) and apoptosis/proliferation ratios (C) are lower in the gastric antrum in persons infected with *cagA*<sup>+</sup> *Helicobacter pylori* strains. Epithelial cell proliferation was scored 1-3 by use of immunoperoxidase staining with Mib-1 anti-Ki-67 antibody by a single pathologist blinded to *H. pylori* genotype. Apoptosis was quantitated by use of the terminal uridine deoxynucleotidyl nick end-labeling (TUNEL) assay and expressed as apoptotic cellular index (number of positively stained epithelial cells/100 epithelial cells counted). The apoptosis/proliferation ratio was determined by dividing the apoptotic cellular index by the proliferation score. *cagA* status of infecting isolates was determined by the serology and polymerase chain reaction of genomic DNA. Each data point reflects averaged scores from two antral biopsy specimens (one data point per patient). Median values with interquartile ranges are shown adjacent to data points.



*H. pylori* *cagA*<sup>+</sup> *vacA* *s1a* strains results in increased proliferation within the gastric antrum without a concordant increase in apoptosis.

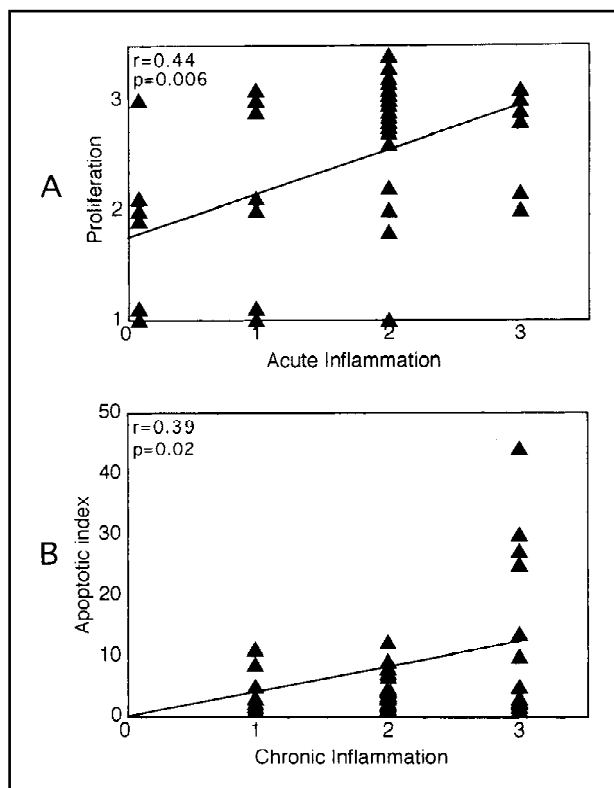
### Epithelial Cell Proliferation and Association With Acute Mucosal Inflammation Caused by *H. pylori* *cagA*<sup>+</sup> Strains

Severity of acute inflammation within the gastric mucosa has been independently related to infection with *cagA*<sup>+</sup> *H. pylori* strains (15,16) and to epithelial cell proliferation (14). Therefore, we directly compared inflammation and proliferation scores for the 20 patients infected with *cagA*<sup>+</sup> strains (Fig. 2, A). Antral biopsy scores of epithelial cell proliferation and acute inflammation were significantly associated in persons harboring *cagA*<sup>+</sup> strains; similar results were found when we examined *vacA* *s1a* strains (data not

shown). No significant correlation was found between scores for proliferation and acute inflammation in persons infected with *cagA*<sup>-</sup> strains or in uninfected persons ( $P = .86$  and  $P = .6$ , respectively), at least in part because inflammation scores were low.

### Epithelial Cell Proliferation and Other Histologic Parameters of Inflammation and Injury Caused by *H. pylori*

To better understand the mechanisms by which cell growth might be linked with tissue injury, we examined the relationship between epithelial cell proliferation and other pathologic markers of gastric inflammation and injury in *H. pylori*-infected persons (Table 1). Except for acute inflammation as noted above, proliferation was not significantly associated with any indices of gastric inflammation and injury in persons infected with either



**Fig. 2.** Association between **A)** proliferation and acute inflammation and **B)** apoptosis and chronic inflammation in persons infected with *cagA*<sup>+</sup> *H. pylori* strains. Epithelial cell proliferation was scored 1-3 by use of immunoperoxidase staining with Mib-1 anti-Ki-67 antibody. Apoptosis was quantitated by using the terminal uridine deoxynucleotidyl nick end-labeling (TUNEL) assay as described in the text. Acute and chronic inflammation was scored 0-3 by a single pathologist blinded to proliferation and apoptosis results. Each data point reflects scores from each antral biopsy (two data points per patient).

*cagA*<sup>+</sup> or *cagA*<sup>-</sup> strains, although a trend toward significance was seen between proliferation and chronic inflammation in the *cagA*<sup>+</sup> group ( $P = .06$ ). When proliferation was examined in relation to histology in *H. pylori*-infected persons stratified by *vacA* signal sequence genotypes, the observations for *s1a* strains mirrored those for *cagA*<sup>+</sup> strains (data not shown). These findings demonstrate that epithelial cell proliferation is significantly linked with severity of acute inflammation only in patients infected with *cagA*<sup>+</sup> and *vacA* *s1a* strains.

### Relationship Between Apoptosis and Histologic Indicators of Inflammation and Injury

According to current models, tissue integrity is maintained when rates of cell growth and loss are matched (25). We, therefore, also sought to determine whether epithelial cell apoptosis was linked to gastric inflammation. Among the persons infected with *cagA*<sup>+</sup> strains, scores for apoptosis and chronic inflammation were significantly associated (Fig. 2, B); no other correlations were significant (Table 1). The findings for *s1a* strains paralleled those for *cagA*<sup>+</sup> strains (data not shown).

### Discussion

Apoptosis is a normal component of epithelial cell turnover in many tissues, including the gastrointestinal tract (26). Tissue integrity is maintained when the rate of cell loss by apoptosis is matched by the rate of new cell production by proliferation. Since hyperproliferation not balanced by cell death may contribute to malignant transformation (25), the dissociation of proliferation from apoptosis

may be an important mechanism of carcinogenesis, as occurs in colonic neoplasia (27,28).

Carriage of *H. pylori* significantly increases risk for the development of adenocarcinoma of the distal stomach (5,6), and infection with *cagA*<sup>+</sup> strains further increases this risk (10,11); therefore, we examined gastric epithelial cell proliferation and apoptosis in biopsy specimens from patients infected with well-characterized *H. pylori* isolates. Our results demonstrate that persons infected with *H. pylori* strains that possess *cagA* have significantly higher rates of epithelial cell proliferation without an increase in apoptosis scores. By examining the relationships between proliferation, apoptosis, and several histologic parameters of inflammation and injury in the same biopsy specimens, we also have identified induction of acute mucosal inflammation as a potential mechanism by which these bacteria stimulate proliferation.

Of interest, the apoptosis/proliferation ratio was not statistically different between *cagA*<sup>+</sup> and uninfected persons (Fig. 1, C). This result was somewhat unexpected, since it would seem logical, based on proliferation and apoptosis data (Fig. 1, A and B), that persons harboring *cagA*<sup>+</sup> strains should have lower apoptosis/proliferation ratios than uninfected persons. Three potential explanations could account for this finding. First, the presence of *cagA*<sup>+</sup> strains may alter the normal relationship between proliferation and apoptosis that exists in uninfected mucosa. For example, persons in the

**Table 1.** Relationship between proliferation and apoptosis and histologic parameters of inflammation and injury in the gastric antrum in *Helicobacter pylori*-infected persons, stratified by *cagA* genotype\*

Histologic parameter‡	P values†			
	Proliferation§		Apoptosis	
	<i>cagA</i> <sup>+</sup> (n = 20)	<i>cagA</i> <sup>-</sup> (n = 9)	<i>cagA</i> <sup>+</sup> (n = 20)	<i>cagA</i> <sup>-</sup> (n = 9)
Acute inflammation	.006	.86	.40	.40
Chronic inflammation	.06	.40	.02	.70
Vacuolation	.52	1.0	.07	1.0
Mucin depletion	.60	.95	.60	.73
Epithelial degeneration	.98	.95	.58	.73
Atrophy	.89	.77	.55	.26
Intestinal metaplasia	.50	1.0	.69	1.0

\**cagA* status determined by serology and polymerase chain reaction of bacterial genomic DNA.

†P values determined by linear regression analysis.

‡Histologic parameters of inflammation and injury scored 0-3 as described in the "Methods" section.

§Proliferation scored 1-3 using Ki-67 immunohistochemistry as described in the "Methods" section.

||Apoptosis quantitated by the terminal uridine deoxynucleotidyl nick end-labeling (TUNEL) assay as described in the "Methods" section.

cagA<sup>+</sup> group with the lowest apoptosis scores may not have the same degree of proliferation as uninfected persons with the lowest apoptosis scores. Second, the wider interquartile ranges seen in uninfected persons suggest that the apoptosis/proliferation ratio may become significantly higher if a larger group of patients is examined. Finally, unidentified factors that may affect apoptosis and proliferation in addition to *H. pylori*, such as cigarette or medication use, may have contributed to this finding.

Gastric mucosal proliferation rates have been reported to be enhanced (13,14) or unaffected (29) by *H. pylori* infection. A potential explanation for the conflicting findings is that *H. pylori* strains may differ in their ability to induce proliferative responses; our results are consistent with this hypothesis. The fact that linkage between proliferation and neutrophilic infiltration was found only in persons harboring cagA<sup>+</sup> strains may reflect the tendency of these strains to induce more severe mucosal inflammation (15,16). Although it is possible that products of polymorphonuclear cells such as reactive oxygen metabolites may stimulate proliferation, alternatively, undefined bacterial factors also may be responsible.

Other pathogenic bacteria, such as *Staphylococcus aureus* and *Escherichia coli*, produce pore-forming proteins that can induce apoptosis (30), but little is known about the induction of apoptosis by *H. pylori*. In a previous study (17), we found increased epithelial apoptosis in approximately 50% of *H. pylori*-infected patients with duodenal ulcers, although the genotype of the infecting *H. pylori* strains was not determined. In this current report, *H. pylori* isolates were stratified by virulence genotype and we have demonstrated that cagA<sup>+</sup> *H. pylori* strains are associated with relatively lower epithelial cell apoptosis scores than cagA<sup>-</sup> strains. This may be related to the finding that tumor necrosis factor- $\alpha$ , a proinflammatory cytokine found to be elevated in gastric mucosa from *H. pylori*-infected persons (31), inhibits apoptosis by activating NF- $\kappa$ B (32-34). Recent work by our laboratory and others (35,36) has shown that cagA<sup>+</sup> *H. pylori* strains are stronger inducers of NF- $\kappa$ B in gastric epithelial cells than are cagA<sup>-</sup> strains, and that inactivation of picB, a gene that is adjacent to cagA, markedly diminishes this induction. Thus,

enhanced NF- $\kappa$ B induction by cagA<sup>+</sup> *H. pylori* strains would, in light of the new findings, be predicted to result in less apoptosis.

A proposed model for enhanced cancer risk in persons infected with *H. pylori* cagA<sup>+</sup> vacA s1a strains is that such strains lead to increased acute inflammation within the gastric mucosa that drives enhanced epithelial cell proliferation without an increase in epithelial cell apoptosis. This combination of increased antral proliferation and unaltered apoptosis may contribute to the heightened retention of mutagenized cells and subsequent risk for the development of carcinoma.

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## Notes

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## Identification of Tobacco-Specific Carcinogen in the Cervical Mucus of Smokers and Nonsmokers

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**Background:** In 1996, an estimated 15 700 new cases of cancer of the uterine cervix and 4900 deaths from this disease were expected to occur in the United States. In a recent international study, human papillomavirus DNA was found in more than 90% of cervical tumor specimens examined, irrespective of the nationality of the patients from whom the samples were obtained. Although infection with human papillomavirus is the major known risk factor for the development of cervical cancer, it alone is not sufficient. Other etiologic factors that have been associated with this disease include deficiencies in micronutrients, lower socioeconomic status, oral contraceptive use, and cigarette smoking. Several compounds from cigarette smoke (nicotine and its major metabolite, cotinine) have been identified in cervical mucus, and the occurrence of smoking-related DNA damage in the cervical epithelium has been documented. **Purpose:** This investigation was conducted to determine for the first time whether carcinogenic tobacco-specific *N*-nitrosamines are present in the cervical mucus of cigarette smokers and of nonsmokers (most likely as a result of environmental exposure). **Methods:** Cervical mucus specimens from 15 smokers and 10 nonsmokers were subjected to supercritical fluid extraction with the use of carbon dioxide that contained 10% methanol, and the resultant extracts were analyzed for tobacco-specific nitrosamines by use of a very sensitive method that involved gas chromatography and mass spectroscopy analyses. **Results:** In a total of 16 samples obtained from 15 women who were current smokers (two samples from the same woman), we detected the tobacco-

specific nitrosamine 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) at concentrations that ranged from 11.9 to 115.0 ng/g of mucus. Only one of a total of 10 cervical mucus specimens obtained from 10 women who claimed to be nonsmokers did not contain detectable NNK, and NNK concentrations ranged from 4.1 to 30.8 ng/g of mucus in the specimens from the remaining nine women. The concentrations of NNK in specimens from cigarette smokers were significantly higher than those from nonsmokers (mean  $\pm$  standard deviation: 46.9  $\pm$  32.5 ng/g of mucus versus 13.0  $\pm$  9.3 ng/g of mucus; two-tailed Student's *t* test, *P* = .004). **Conclusion:** The cervical mucus of cigarette smokers contains measurable amounts of the potent carcinogen NNK. This compound represents the first tobacco-specific carcinogen identified in this physiologic fluid of women who smoke cigarettes. The presence of NNK in the cervical mucus of nonsmokers is likely due to environmental exposure or to the fact that some of the subjects in this study may not have revealed that they occasionally smoked cigarettes. **Implications:** The presence of NNK in human cervical mucus further strengthens the association between cervical cancer and tobacco smoking. [J Natl Cancer Inst 1997;89:868-73]

Cervical cancer is the leading cause of cancer deaths and the most common cancer among women in developing countries (1). In the United States, it is the third most common cancer among Hispanic women and the sixth most common cancer among Caucasian women (2). In the United States in 1996, there were an estimated 15 700 new cases of cervical cancer and 4900 deaths from this disease (3). The incidence and mortality rates of

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