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Effect of *Helicobacter pylori* on Gastric Epithelial Cell Migration and Proliferation In Vitro: Role of VacA and CagA

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***Helicobacter pylori* infection is associated with inflammation of the gastric mucosa and with gastric mucosal damage. In this study, we sought to test the hypothesis that two *H. pylori* virulence factors (VacA and CagA) impair gastric epithelial cell migration and proliferation, the main processes involved in gastric mucosal healing in vivo. Human gastric epithelial cells (MKN 28) were incubated with undialyzed or dialyzed broth culture filtrates from wild-type *H. pylori* strains or isogenic mutants defective in production of VacA, CagA, or both products. We found that (i) VacA specifically inhibited cell proliferation without affecting cell migration, (ii) CagA exerted no effect on either cell migration or proliferation, and (iii) undialyzed *H. pylori* broth culture filtrates inhibited both cell migration and proliferation through a VacA- and CagA-independent mechanism. These findings demonstrate that, in addition to damaging the gastric mucosa, *H. pylori* products may also impair physiological processes required for mucosal repair.**

Helicobacter pylori is the major causative agent of chronic superficial gastritis and plays a central role in the etiology of peptic ulcer disease (2, 28). Mounting evidence suggests that infection with *H. pylori* increases the risk for development of gastric cancer (29, 30), and recently *H. pylori* has been designated a class I carcinogen by the World Health Organization (45). The mechanisms whereby *H. pylori* exerts its pathogenetic action are not yet well understood. Several virulence factors have been proposed for *H. pylori* (13, 22, 38): motility, adhesion to the gastric epithelium, endotoxin-like activity of the lipopolysaccharide, urease activity, proteolytic enzymes, and phospholipase A.

In addition to the above factors, which are common to all clinical isolates, two factors are produced by only 50 to 60% of wild-type isolates of *H. pylori*: (i) an approximately 90-kDa vacuolating cytotoxin (VacA) which is toxic to epithelial cells in vitro and in vivo (8, 17, 39) and (ii) a 120- to 140-kDa immunodominant protein (CagA) (7, 9, 42). Essentially all *H. pylori* strains possess *vacA*, but those that have the cytotoxin phenotype are referred to as Tox^+. In contrast, only about 60% of wild-type *H. pylori* strains possess *cagA*; all of these are $CagA^+$. An increasing body of evidence suggests that Tox^+ $CagA^+$ *H. pylori* strains are preferentially associated with the development of peptic ulcer disease and gastric cancer (3, 10, 38, 46).

Gastric mucosal defense can be defined in terms of the ability of the gastric mucosa to resist injury, as well as its capacity to respond appropriately to injury so that tissue damage is limited and the survival of the organism is not compromised (44). The ability of the gastric mucosa to repair itself rapidly after damage involves two different processes (19, 36). First, viable surface mucous cells and mucous neck cells migrate from areas adjacent to the injured surface to cover the denuded area and, thus, reestablish epithelial continuity (i.e., restitution). Second, lost cells are replaced by cell division at the proliferative zone in the neck of the gastric gland. The hypothesis of this study was that *H. pylori*, in addition to causing gastric mucosal damage (2, 13, 15, 22, 23), also may impair the process of gastric mucosal healing. This study was designed to determine whether *H. pylori* affects the processes of gastric epithelial cell migration and proliferation and whether VacA or CagA is specifically involved in such a pathogenetic mechanism.

To study these effects, we have used the urease-positive Tox^+ $CagA^+$ wild-type *H. pylori* 60190 (ATCC 49503) and isogenic mutants in which *vacA*, *cagA*, or both genes were disrupted by insertional mutagenesis (11, 17, 43). For comparative purposes we also used *H. pylori* CCUG 17874 (Tox^+ $CagA^+$) (from the culture collection of the University of Göteborg, Göteborg, Sweden), G21 (Tox^- $CagA^-$) (a gift from N. Figura, Siena, Italy), and Bx2 U^+ (Tox^- $CagA^-$) and its urease-negative mutant Bx2 U^- (provided by F. Mégraud, Bordeux, France) (Table 1). All of these strains except Bx2 U^- are wild-type clinical isolates. Bacteria were grown in brucella broth, supplemented with 5% fetal calf serum (Gibco, Grand Island, N.Y.), for 24 to 36 h at 37°C in a thermostatic shaker under microaerobic conditions. As previously described (32), when the bacterial suspensions reached 1.2 optical density units at 450 nm, bacteria were removed by centrifugation and the supernatants were sterilized by passage through a 0.22-μm-pore-size cellulose acetate filter (Nalge Co., Rochester, N.Y.) to obtain the broth culture filtrates. Uninoculated broth filtrate served as a control. To remove ammonia and urea, aliquots of both *H. pylori* broth culture filtrates and control filtrate were dialyzed against Hanks' balanced salt solution for 36 h in di-

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TABLE 1. Effects on cell migration and proliferation of *H. pylori* broth culture filtrates and controls[a]

Sample[b]	Undialyzed		Dialyzed	
	Cell migration[c]	[^{3}H]thymidine uptake (cpm/well)	Cell migration[c]	[^{3}H]thymidine uptake (cpm/well)
Control	29.7 ± 5.1	2,788 ± 311	27.0 ± 4.2	5,404 ± 437
Control plus 4 mM NH_4Cl	ND[d]	ND	23.5 ± 5.0	4,205 ± 596
Broth culture filtrate				
CCUG 17874 (U^+ Tox^+ $CagA^+$)	10.5 ± 5.4[e]	251 ± 12[e,f]	26.0 ± 5.5	1,075 ± 46[e,f]
G21 (U^+ Tox^- $CagA^-$)	13.2 ± 3.2[e]	535 ± 43[e]	28.5 ± 4.9	4,364 ± 454
$Bx2U^+$ (U^+ Tox^- $CagA^-$)	12.8 ± 3.6[e]	625 ± 59[e]	30.5 ± 3.5	5,825 ± 499
$Bx2U^-$ (U^- Tox^- $CagA^-$)	13.5 ± 4.0[e]	563 ± 51[e]	27.8 ± 4.8	6,050 ± 841

[a] The data are means ± SEM for three independent experiments.
[b] Control, uninoculated broth filtrate; U, urease.
[c] Number of cells across the wound edge per centimeter.
[d] ND, not determined.
[e] $P < 0.05$ versus the control.
[f] $P < 0.05$ versus all other broth culture filtrates.

alysis tubing with a 12-kDa-molecular-mass cutoff (Sigma, St. Louis, Mo.). The presence or, if appropriate, the absence of VacA and/or CagA in each filtrate was verified by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with anti-VacA or anti-CagA polyclonal antisera (8, 10).

We have used monolayers of MKN 28 cells as an experimental model. This cell line, of human gastric origin (18), demonstrates gastric-type differentiation and mucous granule production (31, 34). MKN 28 cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air in Dulbecco's modified Eagle's medium-Ham's nutrient mixture F-12 supplemented with 10% fetal calf serum and 1% antibiotic-antifungal solution (both from Gibco) in 60-mm petri dishes (Corning Glass Works, Corning, N.Y.) for the wounding assay and in 24-well dishes (Corning) for the proliferation studies.

To assess cell migration, confluent monolayers of MKN 28 cells were treated with mitomycin (2 μg/ml for 2 h) (12). This dose of mitomycin in the absence of serum virtually abolishes MKN 28 cell growth, causing a 95% decrease in [^{3}H]thymidine uptake compared with MKN 28 cells incubated in medium supplemented with 10% fetal calf serum ($P < 0.001$) (data not shown). Monolayers were then wounded with a single-edge razor blade; three wounds 10 to 12 mm across and separated by 1.5 cm were made in each dish as previously described (6). After wounding, cells were washed with fresh serum-free medium, and the wounded monolayers then were incubated for 24 h with *H. pylori* broth culture filtrates or control filtrates diluted 1:3 in serum-free medium. After fixation in absolute methanol and staining with hematoxylin and eosin, migration was assessed in a blinded fashion to avoid observer bias by counting the number of MKN 28 cells that crossed the wound border. Results are expressed as mean numbers of cells that migrated per centimeter in six 1-cm wound segments within each of triplicate dishes.

Cell proliferation was assayed essentially as previously described (6). Briefly, MKN 28 cells were seeded onto 24-well dishes (2×10^4 per well). Twelve hours after seeding, the serum-containing medium was removed and replaced with serum-free medium so as to synchronize cell cycles. Twelve hours later, cells were incubated for 24 h with broth culture filtrates or control filtrates diluted 1:3 in medium with dialyzed serum. Four hours before the end of the incubation, [^{3}H]thymidine (Amersham International, Little Chalfont, United Kingdom) (1.8 μCi per well) was added. At the conclusion of the incubation period, cells were washed three times with ice-cold phosphate-buffered saline, 10% trichloroacetic acid was added, and the precipitate was passed through glass microfiber filters (Whatman 934 AH) and washed with 100% ethanol. Filters then were transferred to vials containing 10 ml of scintillation cocktail (Econofluor; DuPont de Nemours Italiana, NEN Products, Cologno Monzese, Italy) and counted in a Beckman beta counter. [^{3}H]thymidine uptake was expressed in counts per minute per well. Viability of cells treated with *H. pylori* broth culture filtrates and that of cells treated with the control (uninoculated) filtrate were comparable, as assessed by the trypan blue dye exclusion test (data not shown). Also, we did not detect any cell detachment following incubation of cells with any of the test materials.

All data were expressed as means ± standard errors of the means (SEM) for three independent experiments. The statistical significance of the differences was evaluated by the analysis of variance followed by Newman-Keuls's Q test (37).

Undialyzed broth culture filtrates from Tox^+ $CagA^+$ *H. pylori* 60190 and its isogenic Tox^-, $CagA^-$, or Tox^- $CagA^-$ mutants each induced significant ($P < 0.05$) inhibition of MKN 28 cell migration after experimental wounding (Fig. 1A). In contrast, none of the dialyzed broth culture filtrates had any effect on cell migration (Fig. 1B). The above findings indicate that (i) neither VacA nor CagA alters cell migration after experimental wounding and (ii) undialyzed broth culture filtrates from *H. pylori* 60190 contain a low-molecular-mass (<12-kDa) component that is required for inhibition of cell migration.

Undialyzed broth culture filtrates from the Tox^+ $CagA^+$ *H. pylori* 60190 and its isogenic mutants (Tox^-, $CagA^-$, and Tox^- $CagA^-$) each induced significant ($P < 0.05$) inhibition of cell proliferation (Fig. 2A). However, the proliferation-inhibiting effect exerted by broth culture filtrates from the two Tox^+ strains was greater ($P < 0.05$) than that of broth culture filtrates from the two Tox^- mutants (Fig. 2A). The dialyzed broth culture filtrates from the Tox^- mutants had no effect on cell proliferation, whereas the dialyzed broth culture filtrates from the Tox^+ strains substantially ($P < 0.05$) inhibited cell proliferation (Fig. 2B). The above findings suggest that (i) VacA specifically inhibits cell proliferation and (ii) undialyzed broth culture filtrates from *H. pylori* 60190 contain a low-molecular-mass (<12-kDa) component that also contributes to inhibition of cell proliferation. To investigate further whether VacA specifically inhibits cell proliferation, we evaluated

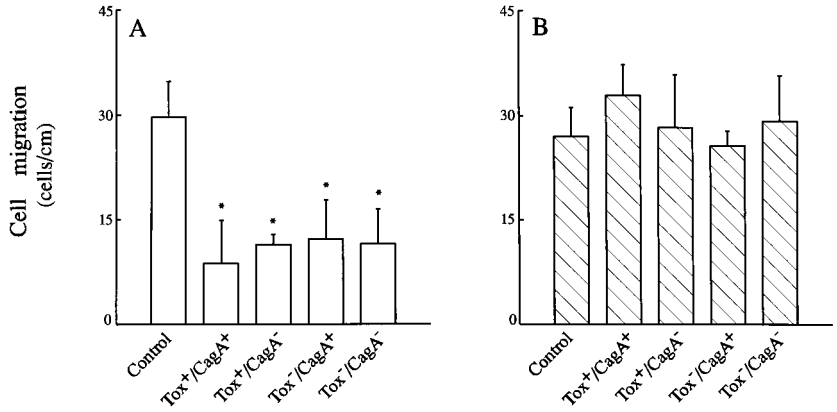

FIG. 1. Effects of broth culture filtrates from wild-type *H. pylori* 60190 (Tox^+ $CagA^+$) and its isogenic mutants (Tox^+ $CagA^-$, Tox^- $CagA^+$, and Tox^- $CagA^-$) on cell migration after experimental wounding. Uninoculated broth filtrates served as negative controls. Cell migration represents the number of cells across the wound edge per centimeter. (A) Undialyzed filtrates; (B) dialyzed filtrates. All filtrates were diluted 1:3 in serum-free medium. Migration of MKN 28 cells incubated with serum-free medium alone was 34.9 ± 7.0 cells per cm (mean ± SEM for three independent experiments) (not shown). All values shown are means ± SEM for three independent experiments. *, $P < 0.05$ versus the control.

whether a neutralizing polyclonal antiserum raised against purified VacA counteracted the inhibition of cell proliferation exerted by dialyzed broth culture filtrates from wild-type *H. pylori* 60190 (Tox^+ $CagA^+$). We found that treatment of broth culture filtrate from the wild-type 60190 strain with anti-VacA rabbit antiserum, but not with nonimmune rabbit serum, significantly ($P < 0.05$) reversed the inhibitory effect on cell proliferation induced by dialyzed broth culture filtrates from the wild-type 60190 strain (Fig. 3).

To determine whether the inhibition of cell migration and proliferation was a peculiarity of *H. pylori* 60190, we evaluated the effects exerted on cell migration and proliferation by other *H. pylori* strains (Table 1). Undialyzed broth culture filtrates from wild-type strains CCUG 17874, G21, Bx2 U^+, and mutant Bx2 U^- each caused significant ($P < 0.05$) inhibition of cell migration, whereas dialyzed broth culture filtrates from these strains failed to induce any effect (Table 1). Undialyzed broth culture filtrates from all the strains also caused a statistically significant ($P < 0.05$) inhibition of cell proliferation. In contrast, dialyzed broth culture filtrate from the Tox^+ strain CCUG 17874 strongly inhibited cell proliferation ($P < 0.05$), whereas dialyzed Tox^- broth culture filtrates failed to induce any effect on cell proliferation (Table 1). That undialyzed broth culture filtrate from strain Bx2 U^- inhibited cell migration and proliferation suggests that urease-dependent ammonia production does not play a role in the inhibition of cell migration and proliferation observed. To further address this point, we evaluated the effect on cell migration and proliferation of the dialyzed uninoculated broth filtrate supplemented with 4 mM NH_4Cl. This concentration of NH_4Cl was similar to the ammonia concentration present when MKN 28 cells were incubated with undialyzed broth culture filtrates from all the urease-positive *H. pylori* strains used (data not shown). We found that neither cell migration nor proliferation was significantly affected by addition of 4 mM NH_4Cl to the dialyzed control (Table 1).

Our data indicate that VacA specifically inhibited cell proliferation without affecting cell migration whereas CagA exerted no effect on either cell proliferation or migration. The studies reported here used a wild-type Tox^+ *H. pylori* strain and an isogenic mutant in which *vacA*, the gene encoding the cytotoxin, had been disrupted by insertional mutagenesis, ren-

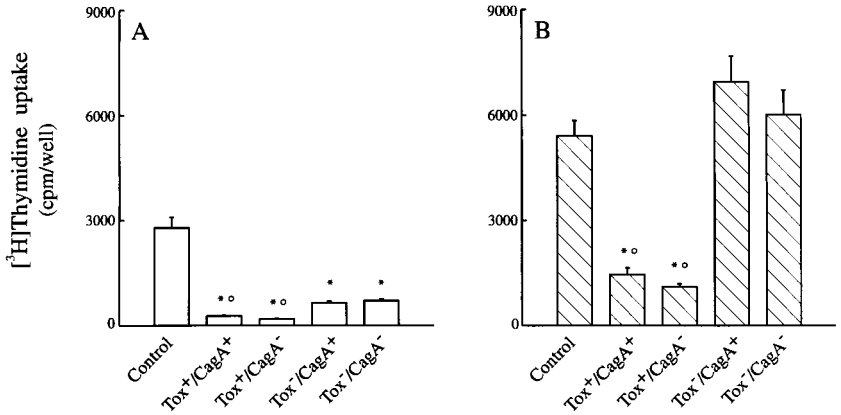

FIG. 2. Effects of broth culture filtrates from wild-type *H. pylori* 60190 (Tox^+ $CagA^+$) and its isogenic mutants (Tox^+ $CagA^-$, Tox^- $CagA^+$, and Tox^- $CagA^-$) on cell proliferation. Uninoculated broth filtrates served as negative controls. (A) Undialyzed filtrates; (B) dialyzed filtrates. All filtrates were diluted 1:3 in medium with dialyzed serum. [^{3}H]thymidine uptake of MKN 28 cells incubated with medium with dialyzed serum alone was 8,395 ± 954 cpm per well (mean ± SEM for three independent experiments) (not shown). All values shown are means ± SEM for three independent experiments. *, $P < 0.05$ versus the control; ○, $P < 0.05$ versus Tox^- broth culture filtrates.

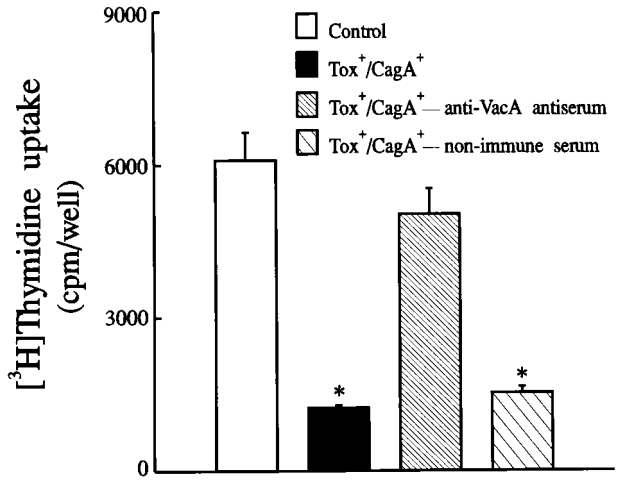

FIG. 3. Effect of immunoneutralization of VacA activity on inhibition of cell proliferation induced by dialyzed broth culture filtrates from wild-type *H. pylori* 60190 (Tox^+ $CagA^+$). Dialyzed broth culture filtrates from the wild-type 60190 strain were treated for 1 h at 37°C with anti-VacA rabbit antiserum or nonimmune rabbit serum at the final serum dilution of 1:20 (8). Dialyzed uninoculated broth filtrate served as the negative control. All filtrates were diluted as for Fig. 2. All values shown are means ± SEM for three independent experiments. *, $P < 0.05$ versus the control.

dering the strain Tox^- (11). The value of these paired strains has been previously reported in studies of cytotoxigenicity (11), ability to induce interleukin-8 secretion by epithelial cells (35), and ability to cause injury to mouse gastric mucosa (17). Moreover, the lack of inhibition of cell proliferation observed with dialyzed broth culture filtrates from the wild-type Tox^- $CagA^-$ G21 or Bx2 U^+ strain provides further evidence that inhibition of cell proliferation was mediated by VacA. The specific role of VacA in the inhibition of cell proliferation is also supported by the immunoneutralization study. However, *H. pylori* filtrates also inhibited both cell migration and proliferation via a mechanism independent of VacA or CagA. In light of our experiments, this effect does not seem to be mediated by ammonia. This is in contrast with a paper by Tsujii et al., who found that gastric cell proliferation and migration were increased by exposure to ammonia in rodents (41). This apparent discrepancy might be due to the fact that Tsujii et al. (41) studied the effect of chronic (8-week) administration of ammonia in vivo while we evaluated the acute effect of ammonia directly on gastric cells in vitro.

Our study represents the first demonstration that VacA directly inhibits gastric epithelial cell proliferation, one of the main processes involved in gastric mucosal healing in vivo. These data are consistent with preliminary observations of other authors (4, 5) who found that dialyzed cell-free supernatants of a VacA-producing *H. pylori* strain either inhibit in vitro proliferation of Kato III cells or delay in vivo healing of acetic acid-induced gastric ulcers in rats. Cell migration and proliferation are essential processes for ulcer healing and for the gastric mucosa to maintain its integrity. Therefore, our findings are relevant to the pathogenesis of *H. pylori*-associated peptic ulcer disease. These findings contrast with the observation that patients with *H. pylori*-associated chronic gastritis have increased bromodeoxyuridine labelling of gastric mucosa compared with normal controls or patients with *H. pylori*-negative gastritis (25). One possible explanation for this apparent discrepancy may be that clinical studies on human subjects infected by *H. pylori* are representative of the effect of persistent *H. pylori* infection whereas the above in vitro and in vivo experimental studies are representative of an acute *H. pylori*-mediated effect. Also, the increased rate of gastric cell proliferation in patients with *H. pylori* infection might be the consequence of increased production of gastrin in vivo (24), which is known to exert a trophic effect on the gastrointestinal mucosa (20). Our in vitro experimental model consists of mucus-producing cells, and therefore, the influence of gastrin is negligible. Moreover, a recent preliminary report suggests that the increased cell proliferation rate in patients with *H. pylori* infection might be related to the *H. pylori*-induced inflammation rather than to a direct action of the pathogen (40). In our in vitro experimental model there are no inflammatory cells, and therefore this hypothesis remains untested. Since the cells used in this study are from an adenocarcinoma, the effects observed potentially could reflect the biology of a tumor cell more than that of a normal, nontransformed cell. Also, since this study was aimed at evaluating the direct effect of *H. pylori* on gastric epithelial cells, we did not study the effect of factors such as extracellular matrix components, which are known to play an important role in the wound healing of gastric mucosa (26).

The mechanisms by which VacA affects gastric cell proliferation are unclear. Mounting evidence suggests that growth factors, especially transforming growth factor α and epidermal growth factor, play an important role in the maintenance of gastric mucosal integrity (1, 21, 33). One possibility is that VacA alters the growth factor-regulated homeostasis of gastric epithelium. This hypothesis is supported by the recent observation (16) that VacA-containing cell-free culture supernatants reduce specific binding of epidermal growth factor to its receptor on Kato III cells in culture, with a consequent reduction of cell proliferative response to epidermal growth factor stimulation. Another hypothesis is that *H. pylori* cytotoxin may inhibit cell proliferation by activating gene products that negatively regulate the cell cycle or induce apoptosis in response to DNA damage (14). To support this hypothesis, a preliminary report has shown that *H. pylori* may cause inflammation-independent apoptosis in vivo (27). Regardless of mechanism, that *H. pylori* infection can cause mucosal injury (2, 13, 15, 22, 23) and inhibit gastric mucosal repair mechanisms provides a strong basis for understanding its role in both ulcer disease and neoplasia.

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