INFECTION AND IMMUNITY, Nov. 1996, p. 4867–4871 Vol. 64, No. 11
0019-9567/96/$04.00+0


# *Helicobacter pylori* Cytotoxin Induces Vacuolation of Primary Human Mucosal Epithelial Cells

PAUL R. HARRIS,[1] TIMOTHY L. COVER,[2] D. RALPH CROWE,[3] JAN M. ORENSTEIN,[4] MARTIN F. GRAHAM,[5] MARTIN J. BLASER,[2] AND PHILLIP D. SMITH[6,7]*

*Division of Pediatric Gastroenterology and Nutrition, Department of Pediatrics,[1] Division of Gastroenterology, Department of Medicine,[6] and Department of Pathology,[3] University of Alabama School of Medicine, and Veterans Affairs Medical Center,[7] Birmingham, Alabama; Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, and Veterans Affairs Medical Center, Nashville, Tennessee[2]; Department of Pathology, George Washington University School of Medicine, Washington, D.C.[4]; and Division of Gastroenterology, Department of Pediatrics, Medical College of Virginia, Richmond, Virginia[5]*

Received 17 May 1996/Returned for modification 25 July 1996/Accepted 9 August 1996

**We investigated whether *Helicobacter pylori* cytotoxin induces vacuolation in primary epithelial cells from normal human mucosa. Epithelial cells purified by enzyme digestion and elutriation were evaluated for vacuolation in a blinded protocol by light and electron microscopy before and after incubation with culture supernatant (CS) from *H. pylori* 60190, which has vacuolating activity for HeLa cells ($Tox^+$), and isogenic *H. pylori* mutant 60190-v1, which lacks this activity ($Tox^-$). Primary epithelial cells (>98% pure) exposed to CS from $Tox^+$ *H. pylori* exhibited marked vacuolation (52% ± 5% of cells) compared with epithelial cells exposed to either CS from $Tox^-$ *H. pylori* (23% ± 3.2%) or uninoculated control broth (23% ± 3.7%) ($P < 0.05$) by light microscopy, which was confirmed by electron microscopy and antibody inhibition studies. These are the first data to show that *H. pylori* cytotoxin causes vacuolation of primary human mucosal epithelial cells.**

*Helicobacter pylori* is the most common bacterial pathogen of the gastrointestinal tract in humans. Infection with this bacterium is associated with chronic gastritis, peptic ulcer disease, and gastric carcinoma (1). However, the mechanisms by which this noninvasive bacterium causes mucosal inflammation are unclear. Particularly perplexing are observations that all infected persons have gastritis but only a small percentage develop serious complications, such as peptic ulcers (13). In this regard, factors that may be associated with the inflammation and damage induced by *H. pylori* include surface proteins (15, 16), urease (12), and cytotoxin (5, 20). *H. pylori* cytotoxin activity was first described by Leunk et al. (14), who showed that supernatants from cultures of approximately 50% of *H. pylori* strains cause vacuolation of eukaryotic cell lines. Subsequently, the *H. pylori* strains that induce vacuolation were shown to be more prevalent in *H. pylori*-infected persons with duodenal ulceration than in infected persons without ulceration (10). To date, characterization of *H. pylori* cytotoxin activity has focused on vacuolation in HeLa cells (5, 14), a transformed cell line that serves as a surrogate for primary epithelial cells. Therefore, to further elucidate the role of the toxin in the pathogenesis of *H. pylori* disease, we investigated the ability of *H. pylori* cytotoxin to induce vacuolation in primary human mucosal epithelial cells.

(This research was presented in part at the Eighth International Meeting of the Society for Mucosal Immunology, San Diego, Calif., 17 to 20 July 1995.)

**Isolation and purification of mucosal epithelial cells.** Segments of normal jejunum from healthy subjects undergoing elective gastrojejunostomy for morbid obesity were used to isolate mucosal epithelial cells in an institutional review board-approved protocol. Pyrogen-free buffers and media were used throughout the procedure, and all instruments and equipment were sterilized prior to use. Briefly, after dissection along the muscularis mucosa, the specimens of jejunal mucosa were rinsed with $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline (0.15 M, pH 7.2; Mediatech, Washington, D.C.) and then incubated twice in Hanks balanced salt solution (Mediatech) plus dithiothreitol (200 mg/ml) at 37°C for 20 min with rotation at 200 rpm to remove residual mucus. To remove the epithelium, the specimens were next incubated twice in Hanks balanced salt solution under the same conditions, except the dithiothreitol was replaced by Dispase (grade 1, 75 μg/ml; Boehringer-Mannheim, Indianapolis, Ind.) (<0.01 ng of endotoxin per ml, as determined by *Limulus* amoebocyte assay). The pooled epithelium was then subjected to counterflow centrifugal elutriation (22), a technique that separates cells on the basis of size and density, for separation into epithelial cell and intraepithelial lymphocyte fractions. The isolated epithelial cells were enumerated by an automated cell counter (Coulter Electronics, Inc., Hialeah, Fla.) and suspended in RPMI 1640 (Mediatech) containing 10% fetal bovine serum (Atlanta Biologicals, Atlanta, Ga.). This procedure yielded $8 \times 10^6$ to $12 \times 10^6$ cells per g of mucosa. Cell purity was evaluated by morphology, ultrastructure, and flow cytometric analysis with fluorescein isothiocyanate-conjugated CD4 and CD8 monoclonal antibodies (Becton Dickinson Immunocytometry Systems, San Jose, Calif.), as previously described (15), to determine whether intraepithelial lymphocytes contaminated the epithelial cell population. Cell viability was assessed by flow cytometric analysis of cells that had been stained with propidium iodide (5 mg/ml), which penetrates and intercalates into the DNA of nonviable cells, causing them to fluoresce red when illuminated with UV light.

**Preparation of *H. pylori* cytotoxin.** *H. pylori* 60190 (ATCC 49503), a well-characterized strain that produces the cytotoxin encoded by *vacA*, and *H. pylori* 60190v-1, an isogenic mutant in which *vacA* has been mutated and, thus, lacking cytotoxin

* Corresponding author. Mailing address: Department of Medicine, Division of Gastroenterology, UAB Station, Birmingham, AL 35294.

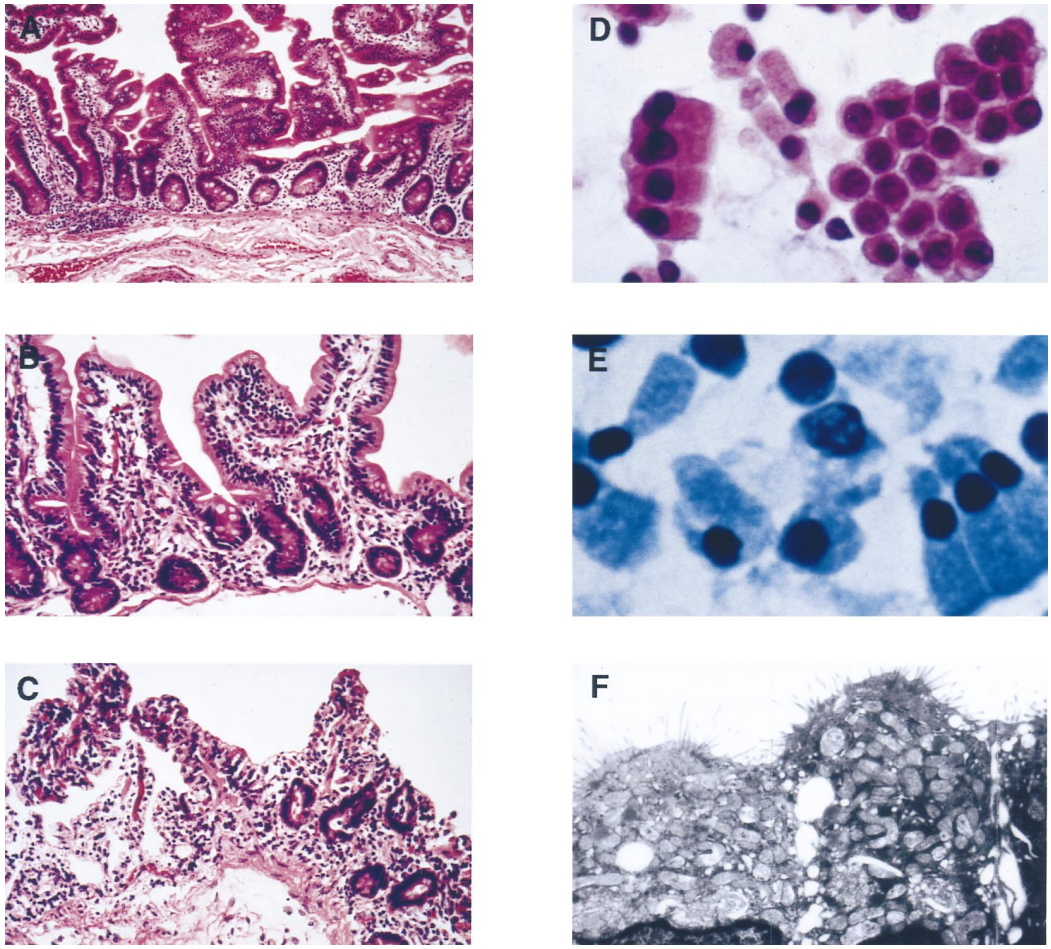


FIG. 1. Isolation of epithelial cells from normal jejunum by enzyme digestion and counterflow centrifugal elutriation. Light microscopy was used to show a typical section of jejunal mucosa and submucosa prior to initiating the isolation procedure (A), mucosa (epithelium, lamina propria, and muscularis mucosa) from which the submucosa has been removed by dissection (B), and lamina propria (C) after dispase removal of the epithelium (D) (small intraepithelial lymphocytes and large epithelial cells). After purification by counterflow centrifugal elutriation, the freshly isolated large cells show the characteristic morphology (E) and ultrastructure (F) of epithelial cells. Magnifications: A, ×30; B, ×30; C, ×30; D, ×60; E, ×125; and F, ×5,000.

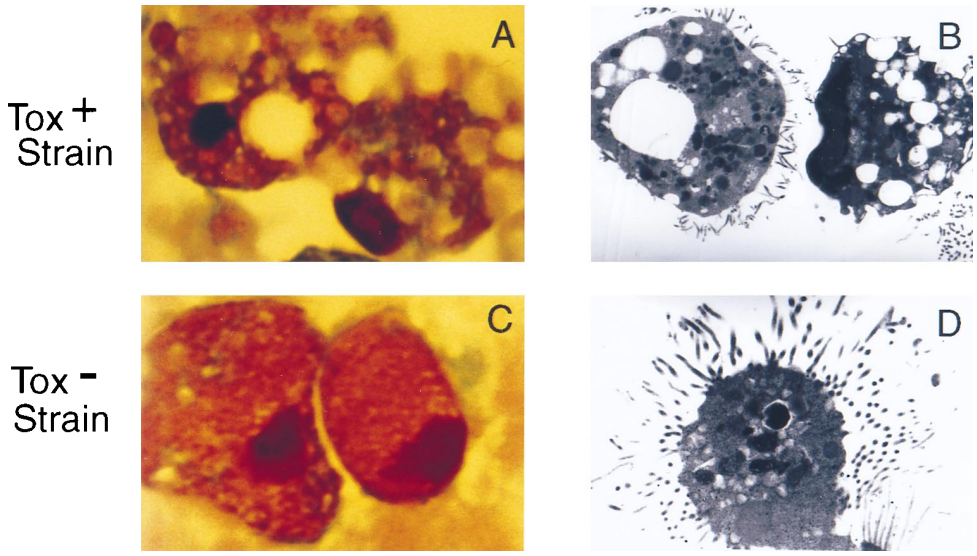


FIG. 4. Mucosal epithelial cells exposed to CS from *H. pylori* $Tox^+$ strain 60190 (A and B) and $Tox^-$ strain 60190v-1 (C and D) and examined by light (A and C) and electron (B and D) microscopy. Multiple large cytoplasmic vacuoles are present in cells exposed to CS from $Tox^+$ *H. pylori* but not in cells exposed to $Tox^-$ CS. Magnifications: A and C, ×500; B and D, ×5,000.

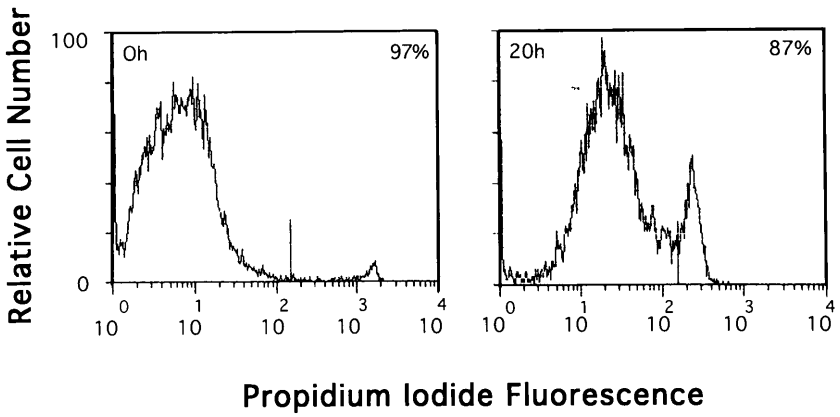


FIG. 2. Epithelial cell viability. Epithelial cells ($10^5$) were incubated with propidium iodide as described in Materials and Methods and immediately assessed by flow cytometric analysis at time zero (freshly isolated cells) and 20 h later to determine the percentage of live cells, which (in contrast to dead cells) do not take up propidium iodide and intercalate it into their DNA.

activity in vitro (8), were studied. Cells were cultured for 3 days under microaerobic conditions at 37°C in brucella broth containing 5% fetal bovine serum. Culture supernatant (CS) was concentrated 30-fold by ultrafiltration and sterilized by passage through 0.2-mm-pore-size cellulose acetate filters (Life Science Products, Denver, Colo.) (3). The presence of vacuolating cytotoxin in CS from *H. pylori* 60190 was confirmed by incubating serially diluted CS with HeLa cells for 18 to 24 h and assaying the cells for vacuolation by neutral red uptake, as previously described (4, 7). The stock CS used in this study had a titer of 1:80.

**Vacuolation assay.** HeLa cells ($10^4$ per well) in Dulbecco's modified Eagle's medium (Mediatech) or primary mucosal epithelial cells ($10^5$ per well) in RPMI plus F12 and 10% fetal bovine serum were incubated in 96-well plates for 4 h at 37°C in 5% $CO_2$ with either (i) CS derived from *H. pylori* strains, (ii) uninoculated brucella broth, or (iii) Dulbecco's modified Eagle's medium alone. Vacuolation in HeLa cells was quantitated spectrophotometrically by neutral red uptake assay as previously reported (4, 7). Because the neutral-red uptake assay requires several manipulations of the cells that impair epithelial cell viability, vacuolation was quantitated by light microscopy, as described below, and then confirmed by electron microscopy. After incubation with test materials or medium, Cytofuge preparations of the epithelial cells were fixed overnight at 37°C in 95% ethanol, treated with Papanicolaou stain, and examined by light microscopy. Viable cells were defined as cells having well-defined cytoplasmic and nuclear outlines, and vacuolation was defined as the presence of one or more intracytoplasmic vacuoles in morphologically viable epithelial cells. For each determination, 100 epithelial cells were counted and the percentage of vacuolated cells was enumerated. Each slide was read three times on different days in a blinded protocol, and the percentages of vacuolated cells were expressed as the means ± the standard errors of the means and compared by Student's *t* test. Parallel specimens were processed by overnight fixation in Carson-Milonig fixative at 37°C, embedded in plastic, and examined with a Philips EM12 electron microscope.

**Inhibition studies.** The ability of *H. pylori* cytotoxin to induce vacuolation of primary human epithelial cells was assayed before and after the CS had been incubated for 1 h with rabbit antiserum (1:20) that was specific for purified vacuolating cytotoxin from *H. pylori* 60190 (3) or with nonimmune serum from a control rabbit. The percentages of vacuolated cells, in triplicate, from three separate experiments were expressed as the means ± the standard errors of the means and compared by Student's *t* test.

**Epithelial cell characteristics.** Progressive steps in a routine isolation of epithelial cells are shown in Fig. 1. From normal jejunum (Fig. 1A), submucosa is removed by dissection (Fig. 1B), after which the epithelium is dissociated from the lamina propria (Fig. 1C) by neutral protease digestion. The epithelium (epithelial cells and intraepithelial lymphocytes) (Fig. 1D) is next separated by counterflow centrifugal elutriation into highly purified populations of intraepithelial lymphocytes (elutriation flow rate, 9.0 to 10.3 ml/min) and epithelial cells (elutriation flow rate, 23.5 to 24.5 ml/min) (Fig. 1E). Flow cytometric analysis revealed that <1% of the cells were $CD4^+$ and $CD8^+$ cells (data not shown). Light microscopy (Fig. 1E) showed that the cells had epithelial cell features, including relatively large size, a columnar configuration, and the presence of cytoplasmic granules (lysosomes), and that they were highly purified. Electron microscopy (Fig. 1F) of freshly isolated cells confirmed these epithelial cell features and also demonstrated densely packed microvilli. Cell viability, as assessed by propidium iodide staining, was routinely >95% immediately after purification and >80% after 20 h of culture (Fig. 2). Taken together, these observations indicate that the epithelial cells obtained by this procedure were highly purified, but viability was reduced after 20 h of culture.

***H. pylori* cytotoxin induces vacuolation of primary human epithelial cells.** The decline in the viability of primary human epithelial cells after 20 h of culture necessitated assaying for the effect of *H. pylori* cytotoxin on the cells much earlier than the 18- to 24-h time point typically used in neutral red uptake assays involving HeLa cells (4, 7). Therefore, we first showed that HeLa cells exposed for only 4 h to CS from an *H. pylori* $Tox^+$ strain exhibited marked vacuolation (>50% of the exposed cells) by neutral red uptake assay (data not shown), indicating that an assay based on 4 h of exposure could detect vacuolating cytotoxin activity. We next analyzed primary human epithelial cells for morphological evidence of vacuolation after 4 h of exposure to the cytotoxin. As shown in Fig. 3, 52% ± 5% of the epithelial cells exposed to $Tox^+$ CS contained one or more vacuoles by light microscopy, compared with 23% ± 3.2% of epithelial cells exposed to $Tox^-$ CS or 23% ± 3.7% of cells exposed to broth alone ($P < 0.05$). Evidence of significantly more epithelial cell vacuolation in the presence of CS from $Tox^+$ *H. pylori* than in CS from the isogenic *vacA*$^-$ strain

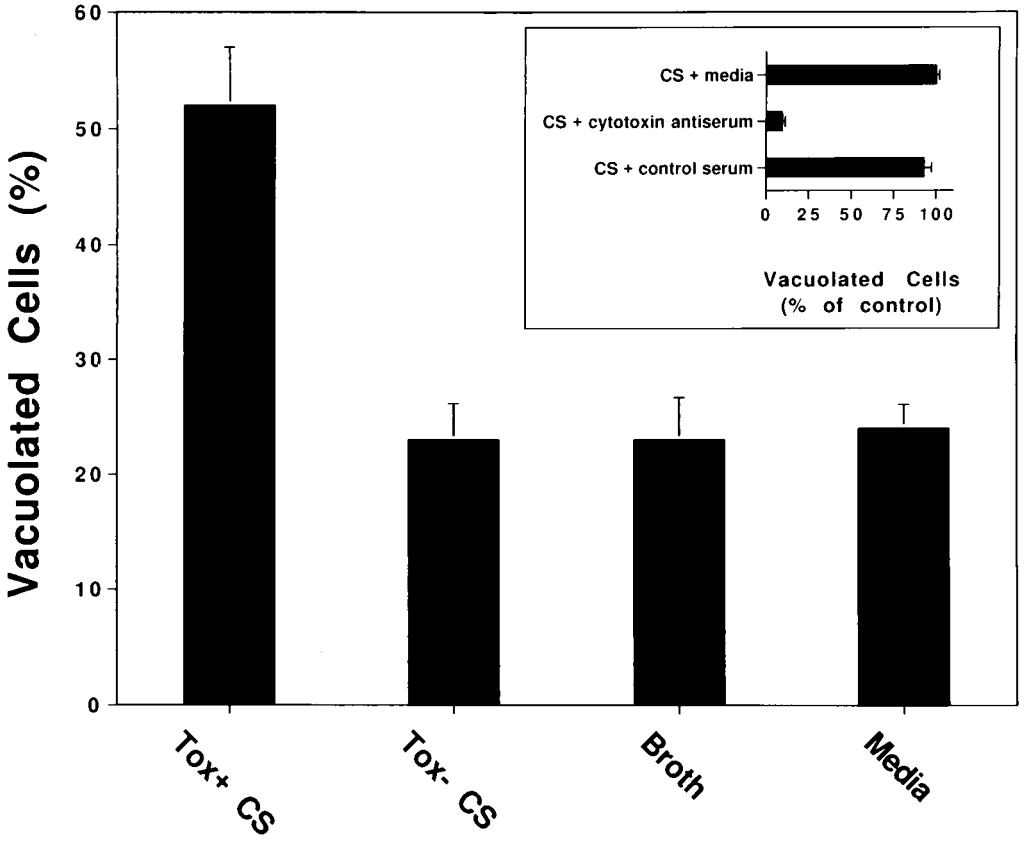


FIG. 3. Vacuolation of primary mucosal epithelial cells induced by *H. pylori* cytotoxin. Primary human epithelial cells were incubated for 4 h with *H. pylori* CS (1:10 dilution) from $Tox^+$ strain 60190, $Tox^-$ strain 60190v-1, uninoculated control broth, or medium alone and then evaluated by light microscopy for the presence of vacuoles. Each value is the mean ± the standard error of the mean (error bar) of three separate determinations from a representative experiment ($n = 3$). (Inset) Effect of cytotoxin antiserum versus control serum (1:20 dilution) on *H. pylori* cytotoxin-mediated vacuolation of primary human epithelial cells.

was confirmed by electron microscopy (Fig. 4). Taken together, these data show that *H. pylori* cytotoxin induced vacuolation in primary human epithelial cells.

**Specificity of cytotoxin-induced vacuolation.** To evaluate the specificity of *H. pylori* cytotoxin in inducing epithelial cell vacuolation, CS from *H. pylori* 60190 was preincubated with rabbit antiserum to purified vacuolating cytotoxin from *H. pylori* 60190 or control normal rabbit serum and then evaluated for vacuolating activity. Antiserum against the purified cytotoxin almost completely reversed cytotoxin-induced vacuolation in primary human epithelial cells ($P < 0.0002$) (Fig. 3, inset), whereas the nonimmune serum had no such effect. These data confirm that the ability of *H. pylori* CS to induce vacuolation is specific for the cytotoxin.

The present study utilizes light and electron microscopy to show that *H. pylori* cytotoxin induces vacuolation in primary human epithelial cells. Antibody inhibition studies confirmed the specificity of cytotoxin-induced vacuolation. This is the first reported observation that the cytotoxin produced by *H. pylori* can cause morphological changes in primary human epithelial cells that resemble the vacuoles demonstrated in transformed cell lines.

Previous studies have shown that supernatants from $Tox^+$ *H. pylori* strains induce vacuolation in many mammalian cell lines, including HeLa, Intestine 407, HEp-2, WiDR, 5637, Vero, and KATO III (5, 20), but HeLa cells have been the preferred cell line in which to study the effect of *H. pylori* cytotoxin because of the availability of the line and the reproducibility of the results (6). We used primary human epithelial cells from the jejunum because this tissue was available to us and because it was from normal, albeit obese, donors. Aside from the presence of more mucin-producing cells in jejunal epithelium, we are unaware of significant functional differences between jejunal and gastric epithelial cells. Although the mucosal epithelial cells used in this study were jejunal in origin, were too fragile to use in the neutral red uptake assay, and showed declining viability after several hours of isolation, our results extend earlier observations with transformed cell lines to primary mucosal epithelial cells.

Vacuolating cytotoxin is produced in vitro by approximately 50% of *H. pylori* strains, as determined on the basis of the detection of vacuoles in HeLa cells (5, 20). Although the *vacA* gene encoding the toxin has been cloned (8, 18–20), we used CSs containing functional cytotoxin because at present the *Escherichia coli*-produced recombinant protein is biologically inactive, likely because of incorrect folding (17). In our study, only 50% of the primary cells were vacuolated after exposure to the cytotoxin, reflecting the short (4-h) exposure to the toxin compared with the longer exposures used in previous studies with HeLa cells (7, 14). Nevertheless, the present finding that *H. pylori* cytotoxin induces vacuolation in primary human epithelial cells strengthens the serologic, pathological, and animal model evidence that cell vacuolation is relevant to in vivo events and that $Tox^+$ strains cause epithelial cell damage, resulting in a more aggressive clinical outcome (2, 11, 20, 21). Vacuolation of epithelial cells may contribute to the pathogen-

esis of *H. pylori* infection, resulting in cytokine production (9) and absorption of bacterial components capable of recruiting and activating inflammatory cells (15, 16).

P.R.H. was supported in part by a national scholarship from the Chilean government. P.D.S., M.J.B., and T.L.C. were supported in part by the Medical Research Service of the Department of Veterans Affairs. T.L.C. was also supported in part by NIH grant R29 45293 and by Glaxo, Inc., and P.D.S. was supported by NIH grant DK 47322.

## REFERENCES

1. **Blaser, M. J., and J. Parsonnet.** 1994. Parasitism by the "slow" bacterium *Helicobacter pylori* leads to altered gastric homeostasis and neoplasia. J. Clin. Invest. **94:**4–8.
2. **Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, and R. Rappuoli.** 1993. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc. Natl. Acad. Sci. USA **90:**5791–5795.
3. **Cover, T. L., and M. J. Blaser.** 1992. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. J. Biol. Chem. **267:**10570–10575.
4. **Cover, T. L., P. Cao, C. D. Lind, K. T. Tham, and M. J. Blaser.** 1993. Correlation between vacuolating cytotoxin production by *Helicobacter pylori* isolates in vitro and in vivo. Infect. Immun. **61:**5008–5012.
5. **Cover, T. L., C. P. Dooley, and M. J. Blaser.** 1990. Characterization of and human serologic response to proteins in *Helicobacter pylori* broth culture supernatants with vacuolizing cytotoxin activity. Infect. Immun. **58:**603–610.
6. **Cover, T. L., S. A. Halter, and M. J. Blaser.** 1992. Characterization of HeLa cell vacuoles induced by *Helicobacter pylori* broth culture supernatant. Hum. Pathol. **23:**1004–1010.
7. **Cover, T. L., W. Puryear, G. I. Perez-Perez, and M. J. Blaser.** 1991. Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. Infect. Immun. **59:**1264–1270.
8. **Cover, T. L., M. Tummuru, P. Cao, S. Thompson, and M. J. Blaser.** 1994. Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. J. Biol. Chem. **269:**10566–10573.
9. **Crowe, S., L. Alvarez, M. Dytoc, R. Hunt, M. Muller, P. Sherman, J. Patel, Y. Jin, and P. Ernst.** 1995. Expression of interleukin 8 and CD54 by human gastric epithelium after *Helicobacter pylori* infection in vitro. Gastroenterology **108:**65–74.
10. **Figura, N., P. Guglielmetti, A. Rossolini, A. Barberi, G. Cusi, R. A. Musmanno, M. Russi, and S. Quaranta.** 1989. Cytotoxin production by *Campylobacter pylori* strains isolated from patients with peptic ulcers and from patients with chronic gastritis only. J. Clin. Microbiol. **27:**225–226.
11. **Ghiara, P., M. Marchetti, M. J. Blaser, M. K. R. Tummuru, T. L. Cover, E. D. Segal, L. S. Tompkins, and R. Rappuoli.** 1995. Role of the *Helicobacter pylori* virulence factors vacuolating cytotoxin, CagA, and urease in a mouse model of disease. Infect. Immun. **63:**4154–4160.
12. **Harris, P. R., H. L. Mobley, G. I. Perez-Perez, M. J. Blaser, and P. D. Smith.** 1995. Purified urease from *Helicobacter pylori* induces monocyte/macrophage activation and cytokine production. Gastroenterology **108:**A109.
13. **Hentschel, E., G. Brandstatter, B. Dragosics, A. M. Hirschl, H. Nemec, K. Schutze, M. Taufer, and H. Wurzer.** 1993. Effect of ranitidine and amoxicillin plus metronidazole on the eradication of *Helicobacter pylori* and the recurrence of duodenal ulcer. N. Engl. J. Med. **328:**308–312.
14. **Leunk, R. D., P. T. Johnson, B. C. David, W. G. Kraft, and D. R. Morgan.** 1988. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. J. Med. Microbiol. **26:**93–99.
15. **Mai, U. E. H., G. I. Perez-Perez, J. B. Allen, S. M. Wahl, M. J. Blaser, and P. D. Smith.** 1992. Surface proteins from *Helicobacter pylori* exhibit chemotactic activity for human leukocytes and are present in gastric mucosa. J. Clin. Invest. **175:**517–525.
16. **Mai, U. E. H., G. I. Perez-Perez, L. M. Wahl, S. M. Wahl, M. J. Blaser, and P. D. Smith.** 1991. Soluble proteins from *Helicobacter pylori* activate monocytes/macrophages by a lipopolysaccharide-independent mechanism. J. Clin. Invest. **87:**894–900.
17. **Manetti, R., P. Massari, D. Burroni, M. de Bernard, A. Marchini, R. Olivieri, E. Papini, C. Montecucco, R. Rappuoli, and J. L. Telford.** 1995. *Helicobacter pylori* cytotoxin: importance of native conformation for induction of neutralizing antibodies. Infect. Immun. **63:**4476–4480.
18. **Phadnis, S. H., D. Ilver, L. Janzon, S. Normark, and T. U. Westblom.** 1994. Pathological significance and molecular characterization of the vacuolating toxin gene of *Helicobacter pylori*. Infect. Immun. **62:**1557–1565.
19. **Schmitt, W., and R. Haas.** 1994. Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. Mol. Microbiol. **12:**307–319.
20. **Telford, J. L., P. Ghiara, M. Dell'Orco, M. Comanducci, D. Burroni, M. Bugnoli, M. Tecce, S. Censini, A. Covacci, Z. Xiang, E. Papini, C. Montecucco, L. Parenet, and R. Rappuoli.** 1994. Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. J. Exp. Med. **179:**1653–1658.
21. **Tricottet, V., P. Bruneval, O. Vire, and J. Camilleri.** 1986. *Campylobacter*-like organisms and surface epithelium abnormalities in active, chronic gastritis in humans: an ultrastructural study. Ultrastruct. Pathol. **10:**113–122.
22. **Wahl, L. M., and P. D. Smith.** 1991. Isolation of macrophages/monocytes from human peripheral blood and tissues, p. 7.6.1.–7.6.8. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Marguilies, E. M. Shevach, and W. Strober (ed.), Current protocols in immunology. John Wiley & Sons, New York.

*Editor:* A. O'Brien